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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 1/38, 5/06, 5/16, 5/22, C07K 14/78	A1	(11) International Publication Number: WO 99/53021 (43) International Publication Date: 21 October 1999 (21.10.99)

(21) International Application Number: PCT/AU99/00265

(22) International Filing Date: 9 April 1999 (09.04.99)

(30) Priority Data:
PP 2912 9 April 1998 (09.04.98) AU
PP 6097 23 September 1998 (23.09.98) AU

(71) Applicant (*for all designated States except US*): BRESAGEN LIMITED [AU/AU]; 38–39 Winwood Street, Thebarton, S.A. 5031 (AU).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): BETTESS, Michael, David [AU/AU]; 3 Windarra Court, Belair, S.A. 5052 (AU). RATHJEN, Peter, David [AU/AU]; (AU). RATHJEN, Joy [GB/AU]; 1 Mimosa Avenue, Blackwood, S.A. 5051 (AU).

(74) Agents: TULLOCH, Debra et al.; Freehills Patent Attorneys, Level 47, 101 Collins Street, Melbourne, VIC 3000 (AU).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: CELL DIFFERENTIATION/PROLIFERATION AND MAINTENANCE FACTOR AND USES THEREOF

(57) Abstract

The present invention relates to a biologically active factor capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells. The present invention also relates to methods of using the biologically active factor to produce from pluripotent cells, pluripotent cells having different properties, more particularly EPL cells; and to methods of producing partially or terminally differentiated cells from the pluripotent cells. The present invention also relates to pluripotent cells and partially or terminally differentiated cells, and their uses in human cell and gene therapy, and transgenic animal production.

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CELL DIFFERENTIATION/PROLIFERATION AND MAINTENANCE FACTOR AND USES THEREOF

The present invention relates to a biologically active factor, and more particularly to a factor capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells including embryonic stem (ES) cells. The present invention also relates to methods of using the biologically active factor to produce from pluripotent cells, pluripotent cells having different properties, more particularly EPL cells; and to methods of producing partially or terminally differentiated cells from the pluripotent cells. The present invention also relates to pluripotent cells and partially or terminally differentiated cells, and their uses.

Initial developmental events within the mammalian embryo entail the elaboration of extra-embryonic cell lineages and result in the formation of the blastocyst, which comprises trophectoderm, primitive endoderm and a pool of pluripotent cells, the inner cell mass (ICM/epiblast). As development continues, the cells of the ICM/epiblast undergo rapid proliferation, selective apoptosis, differentiation and reorganisation as they develop to form the primitive ectoderm. In the mouse, the cells of the ICM begin to proliferate rapidly around the time of blastocyst implantation. The resulting pluripotent cell mass expands into the blastocoelic cavity. Between 5.0 and 5.5 d.p.c. the inner cells of the epiblast undergo apoptosis to form the proamniotic cavity. The outer, surviving cells, or early primitive ectoderm, continue to proliferate and by 6.0-6.5 d.p.c. have formed a pseudo-stratified epithelial layer of pluripotent cells, termed the primitive or embryonic ectoderm. The primitive ectoderm gives rise to the germ cells and, during gastrulation, acts as a substrate for the generation of the primary germ layers of the embryo proper and the extra-embryonic mesoderm.

The analysis of developmental potential and differentially expressed genes of temporally distinct pools of pluripotent cells within the pre-gastrulation embryo has identified two distinct populations of pluripotent cells, the ICM and primitive ectoderm. Cells within each population appear homogenous as revealed by transplantation studies and the analysis of gene expression markers. The establishment of pluripotent cell heterogeneity and the determination of sub-

populations of pluripotent cells within the primitive ectoderm, as defined by differential gene expression, has not been documented in embryos prior to formation of the primitive streak at 6.5 d.p.c. A paucity of genetic markers and the small size, complexity and relative inaccessibility of the embryo within the uterine environment between 4.5 and 6.5 d.p.c. has not allowed a comprehensive analysis of pluripotent cell progression.

Pluripotent cells can be isolated from the preimplantation mouse embryo as embryonic stem (ES) cells. ES cells can be maintained indefinitely as a pluripotent cell population *in vitro*, and, when reintroduced into a host blastocyst, can contribute to all adult tissues of the mouse including the germ cells. ES cells, therefore, retain the ability to respond to all the signals that regulate normal mouse development, and potentially represent a powerful model system for the investigation of mechanisms underlying pluripotent cell biology and differentiation within the early embryo, as well as providing opportunities for embryo manipulation and resultant commercial, medical and agricultural applications. Other pluripotent cells and cell lines will share some or all of these properties and applications.

The successful isolation, long term clonal maintenance, genetic manipulation and germ-line transmission of pluripotent cells from species other than rodents has generally been difficult to date and the reasons for this are unknown. International patent application WO97/32033 and US Patent 5,453,357 describe pluripotent cells including cells from species other than rodents, and primate pluripotent cells have been described in International patent applications WO98/43679 and WO96/23362 and in US Patent 5,843,780. However, it would be useful if these pluripotent cells could be transformed into pluripotent cells with different properties.

The differentiation of ES cells can be regulated *in vitro* by the cytokine leukaemia inhibitory factor (LIF) and other gp130 agonists which promote self-renewal and prevent differentiation of the stem cells. However, with the exception of retinoic acid, biological molecules that can induce the differentiation of ES cells

into specific cell types, in the presence or absence of LIF, are currently unknown.

It is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

Applicant has identified a biologically active factor which is capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells including ES cells. More specifically, the factor is capable of causing the transition of pluripotent cells (e.g. ES cells in adherant culture and in suspension culture) to pluripotent cells having different properties, more specifically early primitive ectoderm-like (EPL) cells. Additionally the factor is capable of maintaining and supporting proliferation of these cells *in vitro*. It also allows the isolation and maintenance of EPL cells derived from *in vitro* and *in vivo* primitive ectoderm.

Whilst applicant does not wish to be restricted by theory, it is thought that EPL cells represent an *in vitro* equivalent of the pluripotent cells of a post implantation embryo prior to 6.0 d.p.c. or early primitive ectoderm.

Accordingly, it should be understood that the term "EPL cells" refers to cells derived from pluripotent cells that retain pluripotency and are converted to and/or maintained as cells that express *Oct4* and *Fgf5* by:

(a) the biologically active factor of the present invention or the large or low molecular weight component thereof; or

(b) a conditioned medium according to the present invention; or

(c) an extracellular matrix in the presence or absence of the low molecular weight component according to the present invention.

The pluripotent cells from which the EPL cells may be derived may be but are not restricted to ES cells, *in vitro* or *in vivo* derived ICM/epiblast, *in vitro* or *in vivo* derived primitive ectoderm, teratocarcinoma cells, EC cells, primordial germ cells, EG cells and pluripotent cells derived by dedifferentiation or by nuclear

transfer. EPL cells may also be derived from differentiated cells by dedifferentiation.

Furthermore, the EPL cells may be capable of but are not restricted to reversion to ES cells by the removal of (a), (b) or (c) in the presence of a gp130 agonist.

The biologically active factor preferably includes two components, a low molecular weight component and a large molecular weight component.

The low molecular weight component may be an amino acid or functionally active analogue thereof or a peptide or functionally active fragment or analogue thereof. Preferably the low molecular weight component is proline or a functionally active analogue thereof or a peptide including proline or a functionally active fragment or analogue thereof. Even more preferably the low molecular weight component is L-proline or a peptide including L-proline. The peptide preferably has a molecular weight of less than approximately 5 kD, more preferably less than approximately 3 kD. Alternatively or in addition, the peptide preferably has a size of approximately 1-11, more preferably approximately 1-7, most preferably approximately 1-4 amino acids. Most preferably the peptide is selected from the group consisting of:

Pro-ala
Ala-pro
Ala-pro-gly
Pro-OH-pro
Pro-gly
Gly-pro
Gly-pro-ala
Gly-pro-OH-pro
Gly-pro-arg-pro
Gly-pro-gly-gly
Val-ala-pro-gly
Substance P frag. 1-4 (Arg-pro-lys-pro)
Substance P free acid

(arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-gly-Leu-metOH)

Protease digested (including collagenase digested) collagen fragments;

and functionally active fragments and analogues thereof; and molecules which compete therewith for biological activity.

By the term "functionally active" is meant that the fragment or analogue is capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells including ES cells.

The large molecular weight component may be a polypeptide or protein. Preferably the large molecular weight component has a molecular weight of greater than approximately 10 kD, more preferably between approximately 50-1000 kD, most preferably between approximately 100-500 kD. The large molecular weight component may be in solution or contained in an extracellular matrix.

In a preferred form of this aspect of the invention the large molecular weight component may be an extracellular matrix protein or functionally active fragment or analogue thereof, for example a fibronectin or laminin or functionally active fragment or analogue thereof. In a particularly preferred form the large molecular weight component is a cellular fibronectin. The cellular fibronectin may have a molecular weight of approximately 210 to 250 kD, as measured on a 10% reducing/denaturing polyacrylamide gel.

In a further aspect of the present invention there is provided a composition capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells including ES cells, said composition including a biologically active factor and/or a low molecular weight component and/or a large molecular weight component as hereinbefore described, together with an adjuvant, diluent or carrier.

The biologically active factor may be isolated from a medium conditioned by cultured cells. Alternatively, the components of the biologically active factor may be purified from other sources, synthesised or recombinantly produced.

Accordingly, in a further aspect of the present invention there is provided a conditioned medium capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, or a fraction thereof including medium components below approximately 5 kDa, and/or a fraction thereof including medium components above approximately 10 kDa.

Preferably the conditioned medium includes a biologically active factor as hereinbefore described or the low or large molecular weight component thereof.

Preferably the conditioned medium is prepared by a method as hereinafter described.

Preferably the conditioned medium is prepared using a hepatic or hepatoma cell or cell line, more preferably a human hepatocellular carcinoma cell line such as Hep G2 cells (ATCC HB-8065) or Hepa-1c1c-7 cells (ATCC CRL-2026), primary embryonic mouse liver cells, primary adult mouse liver cells, or primary chicken liver cells, or an extraembryonic endodermal cell or cell line such as the cell lines END-2 and PYS-2. However, the biologically active factor may be isolated from a medium conditioned by liver or other cells from any appropriate species, preferably mammalian or avian. Alternatively, the activity may also be derived by contributions from two or more different conditioned media from cells which express one or the other of the components.

Accordingly, in a further aspect of the present invention, there is provided a method for preparing a conditioned medium capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said method including

providing

cells, and

a cell culture medium;

culturing the cells in the cell culture medium for a time sufficient to produce the conditioned medium; and

separating the cells from the culture medium to provide the conditioned medium.

The cells may be of any suitable type. Preferably the cells are hepatic or hepatoma cells or a hepatic or hepatoma cell line. More preferably they are selected from the group consisting of a human hepatocellular carcinoma cell line, primary embryonic mouse liver cells, primary adult mouse liver cells and primary chicken liver cells. Most preferably a human hepatocellular carcinoma cell line such as Hep G2 cells (ATCC HB-8065) or Hepa-1c-1c-7 cells (ATCC CRL-2026) is used. Alternatively the cells may be endodermal cells, more preferably primary or cultured extraembryonic cells or cell lines such as the endodermal cell lines END-2 and PYS-2.

The cells may be cultured under conditions suitable for their proliferation and maintenance *in vitro*. This includes the use of serum including fetal calf serum and bovine serum or the medium may be serum-free. Other growth enhancing components such as insulin, transferrin and sodium selenite may be added to improve growth of the cells from which the conditioned medium or extracellular matrix may be derived. As would be readily apparent to a person skilled in the art, the growth enhancing components will be dependent upon the cell types cultured, other growth factors present, attachment factors and amounts of serum present.

The cells may be cultured for a time sufficient to establish the cells in culture. By this we mean a time when the cells equilibrate in the culture medium. Preferably the cells are cultured for approximately 3-5 days.

The cell culture medium may be any cell culture medium appropriate to sustain the cells employed. Where the cells are a liver cell or liver cell line, the culture medium is preferably DMEM containing high glucose, supplemented with 10% FCS, 40 μg/ml gentamycin, 1 mM L-glutamine, 37°C, 5% CO_2.

Separation of the cell culture medium from the cells may be achieved by any suitable technique, such as decanting the medium from the cells. Preferably the cell culture medium is clarified by centrifugation or filtration (e.g. through a 0.22 μM filter) to remove excess cells and cellular debris. Other known means of separating the cells from the medium may be employed providing the separation

method does not remove the growth components from the medium.

In a still further aspect of the present invention there is provided a substantially or partially purified extracellular matrix capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells.

Preferably the extracellular matrix includes a biologically active factor as hereinbefore described or the large molecular weight component thereof.

Preferably the extracellular matrix is prepared by a method as hereinafter described.

Accordingly, in a further aspect of the present invention there is provided a method for preparing an extracellular matrix capable of influencing differentiation, proliferation, and/or maintenance of pluripotent cells, said method including providing

cells,

a support substrate, and

a cell culture medium;

culturing the cells in the cell culture medium in the presence of the support substrate for a time sufficient to produce the extracellular matrix; and

separating the cells and the culture medium from the support substrate to provide the extracellular matrix.

The cells may be of any suitable type. Preferably the cells are hepatic or hepatoma cells or a hepatic or hepatoma cell line. More preferably they are selected from the group consisting of a human hepatocellular carcinoma cell line, primary embryonic mouse liver cells, primary adult mouse liver cells and primary chicken liver cells. Most preferably a human hepatocellular carcinoma cell line such as Hep G2 cells (ATCC HB-8065) or Hepa-1c-1c-7 cells (ATCC CRL-2026) is used. Alternatively the cells may be endodermal cells, more preferably primary or cultured extraembryonic cells or cell lines such as the endodermal cell lines END-2 and PYS-2.

The cells may be cultured as in accordance with the conditions described for preparation of conditioned medium. Isolation of the matrix-associated activity involves separation of the cell culture medium and appropriate cells or cell lines from the ECM and support substratum. This may involve washing the matrix with a suitable buffer. Preferably cells are cultured in 0.2% azide, 0.1 mM PMSF in phosphate buffered saline (PBS) for 6-18 hours to kill and detach cells or cultured in 0.5 mM EGTA for 15 minutes to detach cells, with cells and debris removed by further washes with PBS.

Alternatively, an extracellular matrix capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells may be prepared from animal sources such as visceral, parietal or primitive endoderm, or from animal cells and tissues, e.g. placenta. An extracellular matrix may also be prepared by artificial means such as drying down purified or semi purified extracellular matrix components on tissue culture plates.

Applicant has found that biologically active factors prepared using a human hepatocellular carcinoma cell line, primary embryonic mouse liver cells, primary adult mouse liver cells and primary chicken liver cells are capable of influencing differentiation, proliferation and/or maintenance of mouse ES cells. Thus, whilst applicant does not wish to be restricted by theory, it appears that the factor is biologically active across species.

The components of the biologically active factor may be partially or substantially purified. Purification of the components may be carried out by removal of the cells followed by techniques such as cation exchange chromatography, hydrophobic interaction chromatography, anion exchange chromatography, heparin affinity chromatography, size exclusion chromatography, ultrafiltration, normal phase chromatography and/or reverse phase chromatography. Preferably, purification of the components is carried out using FPLC and HPLC chromatographic techniques.

In a preferred form of this aspect of the invention, the low molecular weight component may be purified by a combination of optional fractionation eg.

ultrafiltration, gel filtration chromatography (preferably on a Sepharose G10 column), normal phase chromatography and gel filtration chromatography (preferably on a Superdex peptide gel filtration column); and preferably sequentially in the stated order.

Accordingly, the present invention provides a method for partially or substantially purifying a low molecular weight component of a biologically active factor capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said method including

providing

a source of said low molecular weight component,

a first gel filtration chromatography support,

a normal phase chromatography support, and

a second gel filtration chromatography support;

contacting the source of said low molecular weight component with the first gel filtration chromatography support to produce a first fraction;

contacting said first fraction with the normal phase chromatography support to produce a second fraction; and

contacting said second fraction with the second gel filtration chromatography support to produce the partially or substantially purified low molecular weight component.

Preferably, the method includes the further step of subjecting the source of said low molecular weight component to a fractionation step, e.g. ultrafiltration to obtain a fraction containing components with a molecular weight of less than approximately 3 kD.

In a further preferred form of this aspect of the invention, the large molecular weight component may be purified by a combination of affinity chromatography (preferably heparin sepharose affinity chromatography, e.g on a heparin sepharose CL-6B column), anion exchange chromatography (e.g. using a Resource Q anion exchange column) and gel filtration chromatography (preferably on a Superose 6 gel filtration column); and preferably sequentially in the stated order.

Accordingly, the present invention provides a method for partially or substantially purifying a large molecular weight component of a biologically active factor capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said method including

providing

a source of said large molecular weight component,

a heparin affinity chromatography support,

an anion exchange chromatography support, and

a gel filtration chromatography support;

contacting the source of said large molecular weight component with the heparin affinity chromatography support to produce a first fraction;

contacting said first fraction with the anion exchange chromatography support to produce a second fraction; and

contacting said second fraction with the gel filtration chromatography support to produce the partially or substantially purified large molecular weight component.

Alternatively, the large molecular weight component may be purified by a combination of optional fractionation eg ultrafiltration (e.g. using a Amicon DiaFlo YM10 membrane), anion exchange chromatography (e.g. using a Sepharose Q anion exchange column), hydrophobic interaction chromatography (e.g. using a phenyl sepharose hydrophobic interaction column), heparin affinity chromatography (.e.g. heparin sepharose affinity chromatography eg. using a heparin sepharose CL-6B column) and gel filtration (e.g. using a Superose 6 gel filtration column); and preferably sequentially in the stated order.

Accordingly, the present invention provides a method for partially or substantially purifying a large molecular weight component of a biologically active factor capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said method including

providing

a source of said large molecular weight component,

an anion exchange chromatography support,

a hydrophobic interaction chromatography support,

a heparin affinity chromatography support, and

a gel filtration chromatography support;

contacting the source of said large molecular weight component with the anion exchange chromatography support to produce a first fraction;

contacting said first fraction with the hydrophobic interaction chromatography support to produce a second fraction;

contacting said second fraction with the heparin affinity chromatography support to produce a third fraction; and

contacting said third fraction with the gel filtration chromatography support to produce the partially or substantially purified large molecular weight component.

Preferably, the method includes the further step of subjecting the source of said high molecular weight component to a fractionation step, e.g. ultrafiltration to obtain a fraction containing components with a molecular weight of greater than approximately 10 kD.

Alternatively the large molecular weight component may be purified by gelatin affinity chromatography, preferably gelatin sepharose affinity chromatography, optionally followed by dialysis.

Accordingly, the present invention provides a method for partially or substantially purifying a large molecular weight component of a biologically active factor capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said method including

providing

a source of said large molecular weight component,

a gelatin affinity chromatography support, and

a dialysis system;

contacting the source of said large molecular weight component with the gelatin affinity chromatography support to produce a first fraction; and

subjecting said first fraction to dialysis to produce the partially or substantially purified large molecular weight component.

Preferably, the methods for purification of the large molecular weight component include the further step of subjecting the source of said large molecular weight component to a fractionation step, e.g. ultrafiltration to obtain a fraction containing components with a molecular weight of greater than approximately 10 kD. This step may be included at any appropriate point in the purification procedure.

Preferably the source of the large or low molecular weight component is a partially purified biologically active factor as hereinbefore described, a conditioned medium as hereinbefore described or an extracellular matrix as hereinbefore described.

A conditioned medium as hereinbefore described may be used to derive and maintain pluripotent cells including EPL cells or the medium may be fractionated to yield the biologically active factor or components thereof, which may be added alone or in combination to other media to provide a pluripotent cell, e.g. EPL cell deriving and maintaining medium. Alternatively, partially or substantially purified or synthetic or recombinent forms of the biologically active factor or components thereof may be added to other media alone or in combination to provide a pluripotent cell, (e.g. EPL cell) deriving and maintaining medium. The conditioned medium may be used undiluted or diluted (e.g. approx. 10-80%).

When the low molecular weight component is proline, its concentration in the cell maintaining medium is preferably in the range of approximately 40 μM or greater. When the large molecular weight component is cellular fibronectin its concentration in the cell maintaining medium is preferably in the range of approximately 2 μg/ml or greater. Extracellular matrix from relevant cells and cell lines or extracellular matrix prepared artificially may also be used to provide the large molecular weight component of the biological activity. The conditioned medium, factor or large molecular weight component thereof may also be used to coat culture plates to provide the desired biological activity.

The biologically active factor of the present invention or the high or low

molecular weight components thereof may be used to produce pluripotent cells including EPL cells, not only from rodents, but also from other more commercially important species including humans.

Accordingly, in a further aspect of the present invention there is provided a method of producing and/or maintaining early primitive ectoderm-like (EPL) cells, said method including

providing

pluripotent cells , and

a biologically active factor according to the present invention, or the large or low molecular weight component thereof; or

a conditioned medium according to the present invention, or

an extracellular matrix according to the present invention and optionally the low molecular weight component of the biologically active factor of the present invention; and

contacting the pluripotent cells with the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix, to produce or maintain the EPL cells.

The pluripotent cells may be selected from the group consisting of embryonic stem (ES) cells, *in vivo* or *in vitro* derived ICM/epiblast, *in vivo* or *in vitro* derived primitive ectoderm, primordial germ cells, EG cells, teratocarcinoma cells, EC cells, and pluripotent cells derived by dedifferentiation or by nuclear transfer. EPL cells may also be derived from differentiated cells by dedifferentiation.

The biologically active factor or the large or low molecular weight components thereof or the conditioned medium or the extracellular matrix optionally plus low molecular weight component may be used for the isolation, proliferation or maintenance of EPL cells *in vitro*. EPL cells may be generated in adherent culture or as cell aggregates in suspension culture. The biologically active factor or components of the biologically active factor or the conditioned medium or the extracellular matrix optionally plus low molecular weight component may also be used alone or in combination with other factors to generate

differentiated cells, tissues or organs by techniques known to those skilled in the art. The differentiated cells derived from EPL cells may be used in allo, concordant or xenotransplantation, cell therapy, tissue and organ augmentation or replacement, and gene therapy.

In one form of this aspect of the invention, the pluripotent cells may be contacted with the biologically active factor or the large or low molecular weight component thereof or the conditioned medium or the extracellular matrix optionally plus low molecular weight component in the presence of a gp130 agonist such as the cytokine leukaemia inhibitory factor (LIF) preferably at a concentration of greater than approximately 100 units/ml and more preferably greater than approximately 1000 units/ml. Oncostatin M, CNTF, CT1 or IL6 with the soluble IL6 receptor, and IL11 and other gp130 agonists at equivalent levels may also be used.

In the mouse the pluripotent cells may be ES cells or cells derived from pluripotent cells of ICM/epiblast of embryos or cellular aggregates (embryoid bodies) or primitive ectoderm derived from either embryos or from differentiation *in vitro* of ES cells as embryoid bodies or cellular aggregates. In other species pluripotent cells may be derived from equivalent cell sources at the stages relevant to each species. The source of pluripotent cells from all species may include cells derived from primordial germ cells or teratocarcinomas. In addition pluripotent cells may be derived by dedifferentiation (e.g. by reverting differentiated cells to a pluripotent state), or by application of nuclear transfer techniques, (e.g. when the nucleus of a differentiated or partially differentiated cell is transferred into an oocyte or early embryonic cell). The pluripotent cells may be from any vertebrae including murine, human, bovine, ovine, porcine, caprine, equine and chicken. The cells may be isolated by any method known to the skilled addressee.

In a preferred form of this aspect of the invention, the method includes the further step of:

identifying the EPL cells.

The conversion of pluripotent cells to EPL cells may be assessed by expression of marker genes (RNA transcripts and cell surface markers), cell morphology, cytokine responsiveness and/or by differentiation *in vitro* or *in vivo.*

Marker genes which may be used to assess the conversion of pluripotent cells to EPL cells include known markers such as *Rex1*, *Fgf5*, *Oct4* alkaline phosphatase, *uvomorulin*, *AFP*, *H19*, *Evx1*, *brachyury*, and novel marker genes, identified by the inventors, such as L17, Psc1 and K7. Marker genes down regulated during transition from ES cells to EPL cells include *Rex1*, *L17* and *Psc1*. *Fgf5* and *K7* are up regulated during this transition. Pluripotent cell markers *Oct4*, Alkaline phosphatase and *uvomorulin* are expressed by both ES cells and EPL cells in similar levels. Other genes that are expressed in partially differentiated or differentiated embryonic or extraembryonic lineages such as *AFP*, *H19*, *Evx1* and *brachyury* are not expressed in any ES or EPL cells.

Cytokines and growth factors which act differently on EPL and other pluripotent cells include members of the FGF family (e.g. aFGF, bFGF and FGF4) which induce differentiation of EPL cells but not ES cells, and the TGFβ family, (e.g. Activin A) which induces differentiation of EPL but not ES cells. In addition EPL cells are maintained in culture by levels of LIF approximately 10 fold lower than required for ES cell maintenance.

EPL cell differentiation can be distinguished from that of other pluripotent cells by the rates and proportions of structures and differentiated cell types such as primitive ectoderm, visceral endoderm, nascent mesoderm, cardiac muscle, macrophages and neurons formed within cell aggregates or embryoid bodies. When compared to ES cells EPL cells undergo accelerated formation of late primitive ectoderm, accelerated and increased formation of nascent mesoderm, beating cardiocytes and macrophages, and decreased or absent formation of visceral endoderm and neurons. EPL cells do not contribute to embryo development following blastocyst injection, whereas ES cells and EG cells do. EPL cells do not contribute to embryo development following blastocyst injection, whereas ES cells and EG cells do.

The EPL cells may be maintained in a pluripotent state by culture in the presence of the biologically active factor or components thereof or the conditioned medium or the extracellular matrix optionally plus low molecular weight component, in the presence or absence of additional gp130 agonist until further differentiation, induced by factors, conditions or procedures is initiated.

Accordingly in a further aspect of the present invention there is provided a method of producing partially differentiated and/or terminally differentiated cells, said method including

providing

pluripotent cells, and

a biologically active factor according to the present invention, or the large or low molecular weight component thereof, or

a conditioned medium according to the present invention, or

an extracellular matrix according to the present invention and optionally the low molecular weight component of the biologically active factor of the present invention;

contacting the pluripotent cells with the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix, in the presence or absence of additional gp130 agonist, to produce early primitive ectoderm-like (EPL) cells; and

manipulating the environment of the EPL cells to produce the partially differentiated and/or terminally differentiated cells.

The pluripotent cells may be selected from the group consisting of embryonic stem (ES) cells, *in vivo* or *in vitro* derived ICM/epiblast, in vivo or in vitro derived primitive ectoderm, primordial germ cells, EG cells, teratocarcinoma cells, EC cells, and pluripotent cells derived by dedifferentiation or by nuclear transfer. EPL cells may also be derived from differentiated cells by dedifferentiation.

In a preferred form of this aspect of the invention the environment of the EPL cells may be manipulated by maintenance or removal of the biologically active factor or components thereof or the conditioned medium or the extracellular matrix, in the presence or absence of one or more differentiation agents, e.g.

growth factors. For example, maintaining contact of EPL cells with the conditioned medium, biologically active factor, or components thereof, preferably in cell aggregates grown in suspension, preferably in the presence of a growth factor from the FGF family such as FGF4, may be used to produce an at least partially differentiated cell type equivalent to embryonic neurectoderm, which can differentiate further to a range of neural cell types. The neurectoderm is derived via a cell type equivalent to embryonic ectoderm, which can differentiate further to a range of other ectodermal cell types.

In the absence of the biologically active factor, or components thereof, and preferably in the presence of a member of the FGF family, such as FGF4, a growth factor from the TGF-β family may be added to EPL cells to produce an at least partially differentiated cell type equivalent to embryonic nascent mesoderm, which can differentiate further to a range of mesodermal cell types.

In a further preferred form of this aspect of the invention embryoid bodies or aggregates formed from pluripotent cells in the presence of conditioned medium, or biologically active factor, or components thereof, may be disaggregated to a single cell suspension. When reaggregated in the absence of conditioned medium, or biologically active factor, or components thereof, preferably in the presence of a member of the FGF family (eg FGF4) these cells differentiate predominantly to an at least partially differentiated cell type equivalent to embryonic nascent mesoderm, which can differentiate further to a range of mesodermal cell types including blood and muscle lineages.

The pluripotent cells may be selected from the group consisting of embryonic stem (ES) cells, *in vivo* or *in vitro* derived ICM/epiblast, in vivo or in vitro derived primitive ectoderm, primordial germ cells, EG cells, teratocarcinoma cells, EC cells, and pluripotent cells derived by dedifferentiation or by nuclear transfer. EPL cells may also be derived from differentiated cells by dedifferentiation.

In a further preferred form of this aspect of the invention EPL cell differentiation is achieved by a process of first forming monolayer cultures of EPL cells, and then further culturing the cells in the presence of members of the FGF

family or TGFβ family. Cells derived by this process are differentiated cells with distinctive morphologies.

Furthermore, formation of EPL cell aggregates in suspension culture, in the absence of conditioned medium, or biologically active factor, or components thereof, by aggregation of EPL cells formed in adherent or suspension culture, results in the differentiation of these cells in the absence of cell types, such as visceral endoderm, which are known to influence the differentiation of pluripotent cells in the embryo. Aggregated EPL cells differentiated in this manner form high levels of nascent mesoderm that can be further differentiated to form beating muscle and blood and other tissues of mesodermal origin.

In a further preferred form of this aspect of the invention, the method includes the further step of:

identifying the partially or terminally differentiated cells by procedures including cell surface markers and gene expression markers, morphology and differentiation potential.

Marker genes which may be used to assess the conversion of pluripotent cells to neurectoderm cells and neural lineages include known markers such as *Gbx2*, *Sox1*, *Sox2*, nestin, N-Cam, *Oct4*, *Fgf5* and *brachyury*. Markers down regulated during the transition from pluripotent cells to neurectoderm include *Oct4* and *Fgf5*. Markers up regulated during this transition include *Gbx2*, *Sox1*, *Sox2*, nestin and N-Cam. Markers not expressed during this transition include *brachyury*.

Marker genes which may be used to assess the conversion of pluripotent cells to mesoderm and derivatives include known markers such as *Oct4*, *Fgf5* and *brachyury* and *Nkx 2.5* Markers down regulated during the transition from pluripotent cells to mesoderm include *Oct4* and *Fgf5*. Markers up regulated during this transition include brachyury and, as the mesoderm differentiates into muscle precursors, *Nkx 2.5*.

Mesoderm and ectoderm germ layer cells can be distinguished on the basis

of their differentiation potential in vitro. Mesoderm germ layer can differentiate into muscle and blood lineages but not neurectoderm or neural lineages. Ectoderm germ layer can differentiate into neurectoderm and neural lineages but not muscle and blood lineages.

In a further aspect of the present invention there is provided a method for producing predominantly ectodermal germ layer cells, for producing from them predominantly partially differentiated ectodermal and neurectodermal cells, and also for producing predominantly terminally differentiated ectodermal cells, including but not restricted to dermal cells, and also for producing predominantly terminally differentiated neuronal cells.

There is also provided a method for producing predominantly mesodermal germ layer cells, for producing from them predominantly partially differentiated mesodermal cells, and also for producing predominantly terminally differentiated mesodermal cells, including but not restricted to blood cells and beating cardiocytes.

In a further aspect of the present invention there is provided an ectodermal germ layer cell, a neurectodermal cell, a partially differentiated ectodermal cell or partially differentiated neurectodermal cell, and a terminally differentiated ectodermal cell, such as a dermal cell, and a terminally differentiated neuronal cell produced by the method of the present invention.

In a further aspect of the present invention there is provided a mesodermal germ layer cell, a partially differentiated mesodermal cell, and a terminally differentiated mesodermal cell, such as a blood cell and muscle cell produced by the method of the present invention.

In a further aspect of the present invention there is provided a cultured EPL cell.

Alternatively, the EPL cells may be reverted to ES cells by removal of the conditioned medium, or biologically active factor, or components thereof in the

presence of a gp130 agonist such as LIF preferably at levels of about 1000 units/ml or greater or other gp130 agonists at equivalent levels. This is accompanied by reestablishment of ES cell gene expression, cytokine responsiveness, morphology and differentiation potential, both *in vivo* and *in vitro.* This provides means for generating ES cells by reversion or dedifferentiation of primitive ectoderm or EPL cells from any desired species, and particularly from species where ES cells have not been available previously.

Accordingly, in a further aspect of the present invention, there is provided a method of producing ES cells, said method including

providing

pluripotent cells,

a biologically active factor or large or low molecular weight component thereof according to the present invention, or

a conditioned medium according to the invention, or

an extracellular matrix according to the invention and optionally the low molecular weight component of the biologically active factor according to the present invention; and

a gp130 agonist;

contacting the pluripotent cells with the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix; and in the presence or absence of additional gp130 agonist to produce EPL cells; and

contacting the EPL cells with the gp130 agonist in the absence of the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix; to enable the EPL cells to revert to ES cells.

The pluripotent cells may be of any suitable type, as hereinbefore described.

The method may include the further step of differentiating the ES cells to produce partially or terminally differentiated cells as hereinbefore described.

Preferably the gp130 agonist is leukaemia inhibitory factor (LIF). However, other gp130 agonists such as Oncostatin M, CNTF, CT1, or IL6 with soluble IL6 receptor at levels required for maintaining pluripotent ES cells *in vitro*, may also be used.

In a further aspect of the present invention, there is provided a method of producing genetically modified ES cells, said method including:

providing

pluripotent cells,

a biologically active factor or large or low molecular weight component thereof according to the present invention, or

a conditioned medium according to the invention, or

an extracellular matrix according to the invention and optionally the low molecular weight component of the biologically active factor according to the present invention; and

a gp130 agonist;

contacting the pluripotent cells with the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix in the presence or absence of additional gp130 agonist to produce EPL cells;

modifying one or more genes in the EPL cells; and

contacting the genetically modified EPL cells with the gp130 agonist in the absence of the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium or the extracellular matrix; to enable the genetically modified EPL cells to revert to genetically modified ES cells.

The pluripotent cells may be of any suitable type, as hereinbefore described.

It should be noted that genetically modified ES cells may also be produced according to this method, where the reverted ES cells, and not the EPL cells, are genetically modified.

The method may include the further step of differentiating the genetically modified ES cells to produce genetically modified partially or terminally differentiated cells by methods as hereinbefore described including formation of EPL cells.

Alternatively, genetically modified partially or terminally differentiated cells may be produced by a method which includes

providing

pluripotent cells,

a biologically active factor or large or low molecular weight component thereof according to the present invention, or

a conditioned medium according to the invention, or

an extracellular matrix according to the invention and optionally the low molecular weight component of the biologically active factor according to the present invention; and

a gp130 agonist;

contacting the pluripotent cells with the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix; in the presence or absence of additional gp130 agonist to produce EPL cells;

modifying one or more genes in the EPL cells; and

differentiating the genetically modified EPL cells to produce the genetically modified partially or terminally differentiated cells.

Alternatively partially or terminally differentiated cells may be produced as hereinbefore described and then genetically modified.

The pluripotent cells may be of any suitable type, as hereinbefore described.

The present invention also provides a method of producing genetically modified EPL cells, which method includes

providing

pluripotent cells, and

a biologically active factor or large or low molecular weight component thereof according to the present invention, or

a conditioned medium according to the invention, or

an extracellular matrix according to the invention and optionally the low molecular weight component of the biologically active factor according to the present invention;

modifying one or more genes in the pluripotent cells; and

contacting the genetically modified pluripotent cells with the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix; in the presence or absence of additional gp130 agonist to produce the genetically modified EPL cells.

The pluripotent cells may be of any suitable type, as hereinbefore described.

Alternatively, EPL cells may be produced as hereinbefore described and then genetically modified.

The method may include the further step of differentiating the genetically modified EPL cells to produce genetically modified partially or terminally differentiated cells.

Alternatively genetically modified EPL cells and/or ES cells may be produced by dedifferentiating genetically modified partially or terminally differentiated cells by a method including:

providing

partially or terminally differentiated cells ; and

a biologically active factor or large or low molecular weight component thereof according to the present invention, or

a conditioned medium according to the invention, or

an extracellular matrix according to the present invention and optionally a low molecular weight component of a biologically active factor according to the invention;

modifying one or more genes in the partially or terminally differentiated

cells; and

dedifferentiating the genetically modified partially or terminally differentiated cells, by methods that include contacting the genetically modified dedifferentiated cell with the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix; in the presence or absence of additional gp130 agonist to produce the genetically modified EPL cells.

The genetically modified EPL cells so formed can be reverted to genetically modified ES cells and/or differentiated to form genetically modified partially or terminally differentiated cells as hereinbefore described.

In one aspect of this method, genetically modified partially or terminally differentiated cells can be used as karyoplasts in nuclear transfer to form genetically modified dedifferentiated pluripotent cells. The dedifferentiated pluripotent cells so formed can be used to produce genetically modified ES cells and EPL cells by methods that include contact with

a biologically active factor or large or low molecular weight component thereof according to the present invention, or

a conditioned medium according to the present invention, or

an extracellular matrix according to the present invention and optionally a low molecular weight component of a biologically active factor according to the invention;

in the presence or absence of additional gp130 agonist.

The genetically modified ES and EPL cells so formed can be differentiated to form genetically modified partially or terminally differentiated cells as hereinbefore described.

Modification of the genes of these cells may be conducted by any means known to the skilled person which includes introducing extraneous DNA, removing DNA or causing mutations within the DNA of these cells. Modification of the genes includes any changes to the genetic make-up of the cell thereby resulting in a cell genetically different to the original cell.

Accordingly, this method provides a pluripotent cell or genetically modified pluripotent cell which has been maintained in its pluripotent state or a genetically modified partially differentiated cell which has been maintained in its partially differentiated state and which may be used to form chimeric animals and transgenic animals, including animals generated by nuclear transfer.

In a further aspect of the present invention there are provided ES cells, genetically modified ES cells, EPL cells, genetically modified EPL cells, partially or terminally differentiated cells and genetically modified partially or terminally differentiated cells produced by the methods of the present invention.

In a further aspect of the present invention, there is provided a method of producing a chimeric animal said method including

providing

a pluripotent cell or a genetically modified pluripotent cell according to the present invention, and

a pregastrulation embryo;

introducing the pluripotent cell or genetically modified pluripotent cell into the pregastrulation embryo; and

monitoring chimera forming ability.

Accordingly, in a still further aspect of the present invention there is provided a chimaeric or transgenic animal, including animals derived by nuclear transfer, produced using a cell or genetically modified cell according to the present invention.

The biologically active factor, components thereof, conditioned medium, extracellular matrix, cells and methods of the present invention have a number of applications, including the following:

(1) Source of cytoplasts or karyoplasts in nuclear transplant. For example:

- EPL cells of any origin, and cells derived from EPL cells (ES cells, partially differentiated cells, terminally differentiated cells) may be

used as cytoplasts or karyoplasts in nuclear transfer.

- Genetically modified ES cells, genetically modified EPL cells, and genetically modified partially differentiated and genetically modified terminally differentiated cells may be used as cytoplasts or karyoplasts nuclear transfer.

- EPL cells derived by dedifferentiation of partially differentiated cells or terminally differentiated cells may be used as source of nuclear material for nuclear transfer.

- Genetically modified EPL cells derived by dedifferentiation of genetically modified partially differentiated cells or genetically modified terminally differentiated cells may be used as source of nuclear material for nuclear transfer.

- Unmodified or genetically modified pluripotent cells, partially differentiated cells, terminally differentiated cells, tissues, organs and animals may be derived by nuclear transfer when EPL cells are used as cytoplasts or karyoplasts.

(2) Use in human medicine. For example:

- EPL cells obtained from any source, and preferably their differentiated progeny obtained by programmed or directed differentiation may be used in unmodified form for human cell therapy.

  The preferred mode of use is to use autologously-derived EPL cells and their progeny.

  - Cells programmed to form ectodermal lineages can be used for cell therapy procedures including but not restricted to neuronal and dermal cell therapy procedures. For example cell therapy

using neuronal cells can be used to treat Parkinson's disease.

- Cells programmed for mesodermal lineages can be used for cell therapy procedures including but not restricted to bone marrow and muscle cell therapies; for example, for the treatment of cancer and for bone marrow rescue.

- Genetically modified EPL cells obtained by any means described in this application and preferably their differentiated progeny obtained by directed or programmed differentiation may be used for human gene therapy.

  Such gene therapy would preferably be conducted using autologously-derived EPL cells or their differentiated progeny.

  - Examples of genetic diseases that could be treated include but are not restricted to haemophilia, diabetes type 1, Ducheynne's and other muscular dystrophies, Gauche's disease and other mucopolysaccharide diseases, cystic fibrosis.

- EPL cells obtained from any source, and preferably their differentiated progeny obtained by programmed or directed differentiation may be genetically modified to render them resistant to viral infection by genetic manipulation of for example viral receptors to provide cells conferring resistance to infection by that virus.

- EPL cells obtained from any source, and preferably their differentiated progeny obtained by programmed or directed differentiation may be genetically modified to allow the cells to act as drug delivery systems for biologically active protein drugs such as cytokines or lymphokines for example interleukin-2 for treatment of cancers and other diseases.

- Unmodified or genetically modified EPL cells obtained from any source, and preferably their differentiated progeny obtained by programmed or directed differentiation may be used to generate cells and tissues and components of organs for transplant.

The present invention will now be more fully described with reference to the accompanying examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

In the figures and tables:

Figure 1. MEDII effects the transition of ES cells to EPL cells. ES cells grown in medium containing LIF (A), 50% MEDII + LIF (B) or no additives (C) after 4 days in culture. ES cells cultured in the presence of MEDII show a characteristic morphology associated with the formation of EPL cells. The bar indicates 50 µm. (D) ES cells were seeded at a density of 250 cells/cm^2 in DMEM containing LIF, 50% MEDII+LIF, 50% MEDII and without addition. After 5 days the cultures were stained for alkaline phosphatase and haematoxylin, and the percentage of ES, EPL and differentiated colonies determined. Alkaline phosphatase positive colonies were subdivided into ES and EPL cell colonies on the basis of morphology, differentiated colonies (D) were determined as alkaline phosphatase negative. Plating efficiencies (%) for each condition were 42.8 +/- 8.7 (LIF); 47.26 +/- 2.6 (50% MEDII+LIF); 40.5 +/- 5.9 (50% MEDII); 41.4 +/- 6.6 (no addition).

Figure 2. (A) Northern blot analysis of ES and EPL cell RNA. 20 µg of total RNA, isolated from E14 ES cells, EPL cell derivatives cultured in MEDII or MEDII+LIF for 2, 4, 6, and 16 days (i), and spontaneously differentiated ES cells cultured for 6 days in the absence of exogenous factors (ii), was analysed for the expression of *Fgf5*, *Rex1*, *Oct4*, and *mGAP*. *Fgf5* transcripts were 2.7 and 1.8 kb (Hébert et al., 1990), *Rex1* 1.9 kb (Hosler et al., 1989), *Oct4* 1.55 kb (Rosner et al., 1990) and *mGAP* 1.5 kb. (B,C) In situ analysis of ES (B) and EPL (C) cell layers for the expression of *Oct4* . (D,E) ES (D) and EPL (E) cells were stained for the presence of alkaline phosphatase. (F) 10 µg RNA from ES cells and EPL cells

cultured for 2, 4 and 6 days in MEDII+LIF and MEDII was analysed for the expression of *Gbx2*, *Oct4* and *mGAP* by RNase protection. (G) In situ analysis of EPL cells for the expression of *Fgf5*. (H) 20 µg of total RNA from ES cells and EPL cells maintained in MEDII+LIF for 2 and 6 days was analysed by northern blot for the expression of *uvomorulin* (*Uvo*). *mGAP* expression was used to normalise RNA levels. The bars indicate 32 µm in B-E and 25 µm in F.

Figure 3. Sequence of *L17* (A), *K7* (B) and *Psc1* (C) ddPCR products.

Figure 4. Northern analysis of *L17* (A), *Psc1* (B) and *K7* (C) expression in 5 µg of poly A RNA (*L17* and *Psc1*) or 20 µg total RNA (*K7*) isolated from ES cells and EPL cells, maintained in culture in MEDII + LIF for up to 8 days. Transcript sizes are indicated in the figure. Northern blots were quantified and normalised to the expression of *Oct4*.

Figure 5. In vivo expression of novel marker genes, *L17* (A), *Psc1* (B) and *K7* (C) by wholemount in situ hybridisation analysis of mouse embryos dissected between 3.5 d.p.c. and 5.5 d.p.c.. PE - primitive ectoderm; PC - proamnionic cavity; VE - visceral endoderm.

Figure 6. hLIF is required for the maintenance of EPL cells in culture. ES cells were seeded at a density of 250 cells/cm^2 into DMEM containing 50% MEDII or 50% MEDII+mLIF (1000 units/ml), hLIF (1000 units/ml), anti-hLIF antibodies (10 µg/ml), hLIF (1000 units/ml) and anti-hLIF antibodies (10 µg/ml), mLIF (1000 units/ml) and anti-hLIF antibodies (10 µg/ml), anti-gp130 antibodies (10 µg/ml) or hLIF (1000 units/ml) and anti-gp130 antibodies (10 µg/ml). After 5 days in culture colonies were stained for alkaline phosphatase and the percentage of EPL cell containing colonies (EPL) and differentiated colonies (D) determined. Plating efficiencies (%) for each condition were 32.5 +/- 1.5 (50% MEDII); 39.8 +/- 1.3 (50% MEDII+mLIF); 35.0 +/- 0.2 (MEDII + hLIF); 25.5 +/- 1.0 (MEDII + anti-hLIF antibodies); 28.8 +/- 3.8 (MEDII + hLIF + anti-hLIF antibodies); 40.0 +/- 0.5 (MEDII + mLIF + anti-hLIF antibodies); 36.8 +/- 3.3 (MEDII + anti-gp130 antibodies); 36.3 +/- 2.3 (MEDII + hLIF + anti-gp130 antibodies).

Figure 7. (A) 5 μm sections stained with haemotoxylin:eosin of aggregates formed from ES cells and grown in DMEM (EBs, i) or DMEM:MEDII (EBMs, ii) for 4 days. (B) Aggregates formed from ES cells and grown in DMEM (EBs, i, iii, v) or DMEM:MEDII (EBMs, ii, iv, vi) for 4 days were analysed for expression of *Oct4* (i, ii), *brachyury* (iii, iv) and *Fgf5* (v, vi) by wholemount in situ hybridisation.

Figure 8. 20 μg of total RNA isolated from EBs and EBMs on days 2, 3 and 4 of development, was analysed for the expression of *Fgf5*, *brachyury*, *Oct4*, and *mGAP* by Northern blot analysis. *Fgf5* transcripts were 2.7 and 1.8 kb (Hébert et al., 1990), *brachyury* 2.1 kb (Lake, 1996), *Oct4* 1.55 kb (Rosner et al., 1990) and *mGAP* 1.5 kb.

Figure 9. (A) Northern blot analysis of EPL cells and reverted EPL cells. 20 μg of total RNA from EPL cells, cultured in the presence of MEDII or MEDII+LIF for 2, 4 and 6 days, and their reverted derivatives, reverted in medium containing LIF alone (2^R, 4^R and 6^R), was analysed by Northern blot for the expression of *Fgf5*, *Rex1*, *Oct4* and *mGAP*. (B) Clonal EPL cell lines #1 and #2 were seeded at a density of 250 cells/cm^2 and 500 cells/cm^2 respectively into medium containing mLIF and 50% MEDII + LIF. After 5 days the cultures were stained for alkaline phosphatase and the percentage of ES, EPL and differentiated colonies was determined. Alkaline phosphatase positive colonies were subdivided into ES and EPL cell colonies on the basis of morphology, differentiated colonies (D) were determined as alkaline phosphatase negative. Plating efficiencies (%) were 18.4 +/- 2.62 (clone #1, LIF); 34.8 +/- 4.56 (clone #1, 50% MEDII + LIF); 20.4 +/- 1.71 (clone #2, LIF); 31.16 +/- 2.93 (clone #2, 50% MEDII + LIF).

Figure 10. (A) 20 μg of total RNA isolated from ES cells (day 0) and ES cell EBs on days 1, 2, 3 and 4 of development was analysed for the expression of *Rex1*, *Fgf5*, *Oct4* and *mGAP* by northern blot analysis. (B) The number of alkaline phosphatase positive pluripotent cell colonies derived from culture of single cell suspensions of day 5, 6, 7 and 8 EBs in DMEM supplemented with LIF (grey) or DMEM supplemented with LIF + 50% MEDII (black).

Figure 11. Maintenance of embryo-derived primitive ectoderm in culture requires MEDII. (A) Morphology of 5.5 d.p.c. embryo explants cultured for 5 days in embryo culture medium (LIF/CIV) or embryo culture medium + 50% MEDII (MEDII/CIV). (B) In situ hybridisation analysis of Oct4 expression in 5.5 d.p.c. embryo explants cultured for 5 days in embryo culture medium + 50% MEDII. The same explant is shown in both panels, with additional 4x magnification in the right panel.

Figure 12. (A) Schematic representation of the strategy used for isolation and maintenance of pluripotent cells from the primitive ectoderm of the mouse embryo. (B) Embryonic explants representative of different stages of the isolation procedure. Explants from several different isolation experiments are shown.

Figure 13. Pluripotent cell colonies isolated from the primitive ectoderm of 5.5 d.p.c. embryos (EEPL cells), and established in long term culture on STO feeder layers, were analysed for expression of alkaline phosphatase and *Oct4* by cell staining and in situ hybridisation.

Figure 14. Two soluble factors within MEDII are responsible for the formation of EPL cells from ES cells. sfMEDII was separated via ultrafiltration on a centricon-3 unit into a retained fraction (R) and an eluted fraction (E). ES cells were seeded at a density of 250 cell/cm^2 into medium containing E, R or E + R in the presence of LIF. After 5 days the cultures were stained for alkaline phosphatase and the percentage of ES, EPL (alkaline phosphatase positive) and differentiated colonies (alkaline phosphatase negative; D) was determined. Plating efficiencies were 48.4% (eluted fraction); 41.6% (retained fraction); 49.2% (eluted and retained fractions); 32.8% (mLIF).

Figure 15. Purification of the low molecular weight component of the EPL cell-inducing activity. (A) Fractionation of E by Sephadex G10 gel filtration. (B) Fractionation of active fractions from A by normal phase chromatography. (C) Fractionation of active fractions from B by Superdex peptide gel filtration. Eluted material was detected by absorbance at 215 nm. Chromatographic fractions were assayed for the presence of the low molecular weight component of the EPL cell-

inducing activity. Solid lines beneath each chromatogram indicate fractions containing this activity.

Figure 16. Purification of the large molecular weight component of sfMEDII - protocol 1. Sequential purification of R via anion exchange (A), hydrophobic interaction (B), heparin affinity (C) and Superose 6 gel filtration (D) chromatography. Protein was detected at 280 nm. A solid bar indicates fractions containing the bioactivity. (E) Silver stained reducing SDS 10% acrylamide gel of active fractions from each stage of purification. Samples contained either 5 µg (100KD; Anion ex.; Hydrophobic interaction) or 2 µg (Heparin affinity; Gel filtration) protein.

Figure 17. Purification of the large molecular weight component of sfMEDII - protocol 2. Sequential purification of R by Heparin Sepharose CL-6B (A), anion exchange (B) and Superose 6 gel filtration (C) chromatography. Protein was detected at 280 nm. A solid bar indicates fractions containing the bioactivity. (D) Silver stained reducing SDS 10% acrylamide gel of active fractions from each stage of purification. Samples contained either 5 µg (sfMEDII), 2 µg (Heparin affinity; anion exchange) or 1µg (gel filtration) protein.

Figure 18. Purification of the large molecular weight component of sfMEDII - protocol 3. (A) Purification of sfMEDII by gelatin Sepharose affinity chromatography. Protein was detected at 280 nm. Elution of bioactivity is indicated by the solid bar. (B) Silver stained reducing SDS 10% acrylamide gel of 5 µg protein from sfMEDII and flow fraction of gelatin Sepharose and 1 µg of cellular fibronectin purified from stromal cells (cFN Stromal cells) and gelatin Sepharose purified large molecular weight component from sfMEDII (cFN SFM2).

Figure 19. Morphology of ES cells cultured in (A) human plasma fibronectin (2 µg/ml), (B) human cellular fibronectin (2 µg/ml) and (C) the large molecular weight component of sfMEDII purified by protocol 2 (2 µg/ml) in the presence of 87 µM L-proline.

Figure 20. Western blot of purified human plasma fibronectin (1 μg, 0.5 μg), human cellular fibronectin (1.0, 0.5, 0.25 μg) and the large molecular weight component of sfMEDII purified by protocol 2 (1.0, 0.5, 0.25 μg) probed with a monoclonal antibody (3E2, Sigma) directed against human cellular fibronectin. The positions of protein size markers are indicated.

Figure 21. Components of the EPL cell-inducing activity can be identified in divergent cell types and species. (A) Morphology of ES cells seeded into DMEM + 50% conditioned medium from primary adult mouse or embryonic chick hepatocytes after 3 days in culture. (B) Morphology of ES cells seeded into DMEM + 50% conditioned medium from Hepa-1c1c 7 cells +/- 40 μM L-proline, or DMEM + 50% conditioned medium from END-2 cells +/- 10 μg cellular fibronectin. (C) 5 μg protein from DMEM conditioned by 1, no cells; 2, Hep G2; 3, Hep 3B; 4, Hepa-1c1c 7; 5, END-2; 6, PYS-2 electrophoresed on a 10% reducing SDS polyacrylamide gel. Proteins were visualised by silver staining. The arrow indicates the position of cellular fibronectin monomers.

Figure 22. Morphology of ES and EPL cell EBs at day 4 of differentiation. (A) ES EBs, (B) EPL EBs. (C, D) EBs were sectioned and stained with toluidine-blue; (C) ES EB, (D) EPL EB.

Figure 23. Expression of the pluripotent cell markers *Fgf5*, *Rex1*, and *Oct4* in differentiating ES and EPL cell EBs. Northern blot analysis was carried out on 20 μg total RNA from ES and EPL cell EBs at indicated days of differentiation. Expression of mGAP was used as a loading control.

Figure 24. EPL cells form parietal endoderm but not visceral endoderm when differentiated as EBs. (A) Expression of *SPARC* in EBs derived from ES and EPL cells at days 1-4 of differentiation as determined by northern blot analysis on 20 μg total RNA. (B, C) Sections of day 4 ES (B) and EPL (C) cell EBs subjected to wholemount in situ hybridisation with antisense DIG-labelled *SPARC*-specific riboprobes. (D, E) Wholemount in situ hybridisation of day 4 ES (D, F) and EPL (E) cell EBs with antisense DIG-labelled *AFP*-specific riboprobes. Areas expressing AFP are indicated with arrows. (F) Cross section of an ES cell

expression.

Figure 25. EPL cells regain the ability to form visceral endoderm when mixed with ES cells during EB formation. Day 4 chimaeric EPL(LZ+):ES EBs formed by 1:1 cell mixing were stained for β-galactosidase activity (cells within circled area), sectioned and stained for AFP protein by immunohistochemistry (black).

Figure 26. Enhanced and accelerated mesoderm induction in EPL cell EBs compared to ES cell EBs. (A) Expression of *brachyury, goosecoid* and *mGAP* in ES and EPL cell EBs at day 0-4 of differentiation as determined by Northern blot analysis on 20 μg total RNA. (B-I) Wholemount in situ hybridisation of ES (B,D,F,H) and EPL (C,E,G,I) cell EBs with antisense DIG-labelled riboprobes specific for *brachyury* (B-E) and *Oct4* (F-1) at day 3 (B,C,F,G) and day 4 (D,E,H,I).

Figure 27. Enhanced and accelerated formation of beating cardiocytes in EPL cell EBs compared to ES cell EBs. (A) The percentage of ES and EPL cell EBs exhibiting beating muscle during days 4 to 12 of differentiation. The mean percentage and s.d. were derived from two independent experiments in each experiment n=48. (B) *Nkx2.5* expression in ES and EPL cell EBs at day 4-12 as determined by Northern blot analysis on 20 μg total RNA.

Figure 28. The ability of EPL cells to form neurons depends on the in vitro differentiation regime employed. (A) Percentages of ES and EPL cell EBs forming neurons on days 8, 10 and 12 of differentiation. (B) Percentages of ES and EPL cell RA-treated aggregates containing neurons. The mean percentage and s.d. was derived from four independent experiments. In each experiment n=32

Figure 29. Differentiation of ES and EPL cells in response to growth factors of the FGF and TGFβ families. (A) An EPL cell colony, differentiated in the presence of bFGF, showing the characteristic differentiated morphology of cell type A. (B) The proportions of ES cell (ES), EPL cell (EPL), differentiated cell (D) and mixed EPL and differentiated cell (SD) colonies after culture of ES and EPL cells in the presence or absence of bFGF (10 ng/ml) after 5 days. (C) An EPL cell colony, differentiated in the presence of activin A, showing the characteristic

differentiated morphology of cell type B. (D) The proportions of ES cell (ES), EPL cell (EPL), differentiated cell (D) and mixed EPL and differentiated cell (SD) colonies after culture of ES and EPL cells in the presence or absence of activin A (150 ng/ml) after 5 days.

Figure 30. Reverted EPL cells have a differentiation potential similar to ES cells when differentiated as EBs. (A) Northern analysis on 20 μg total RNA of *Fgf5* and *brachyury* expression in EBs derived from ES, EPL and EPL^R cells at days 1-4 of differentiation. (B) The percentage of ES, EPL and EPL^R EBs exhibiting beating muscle from day 7 to day 12 of differentiation, n=36. (C) The percentage of ES, EPL and EPL^R EBs forming neurons from day 7 to day 12 of differentiation, n=36.

Figure 31. Directed formation of pluripotent cells to neurectoderm via formation of EBMs. (A) EBMs formed from ES cells and grown for 4 days in DMEM + 50% MEDII and a further 3 days in DMEM + 50% MEDII + 20ng/ml FGF4. Distinctive convoluted cell layers of pseudostratified epithelial appearance are formed within the aggregate. (B) Aggregates grown as for A were seeded onto gelatin treated plastic for 16 hours in DMEM + 50% MEDII + 20 ng/ml FGF4 and analysed for the expression of *Oct4* (i), *brachyury* (ii), *Gbx2* (iii) and *Sox1* (iv) by in situ hybridisation.

Figure 32. Expression of neurectoderm and neural stem cell markers in aggregates derived from EBMs. EBMs (day 4) were cultured for 3 days in DMEM + 50% MEDII + 20 ng/ml FGF4 and seeded onto gelatin for analysis of gene expression at days 8, 9 and 10. (A) In situ hybridisation of aggregates with *Gbx2*, *Sox1* and *Sox2* antisense probes. (B) Immunohistochemistry of aggregates on day 9 with antibodies directed against nestin and N-Cam.

Figure 33. Expression of pluripotent cell and neurectoderm markers during programmed formation of neurectoderm from EBMs. (A) 10 μg total RNA from ES cell EBs (IC+B, day 4), ES cell EBs cultured from day 4 in DMEM + 20 ng/ml FGF4 (IC+B/FGF, days 5-8), EBMs (MEDII, day 4), and EBMs cultured from day 4 in DMEM + 50% MEDII + 20 ng/ml FGF4 (MEDII/FGF, days 5-8) was analysed for

expression of *Oct4*, *Fgf5* and *mGAP* by northern blot. (B) RNase protection for *Gbx2* and *Sox1* on 20 μg total RNA from EBMs (day 4) and EBMs cultured in DMEM + 50% MEDII + 20 ng/ml FGF4 (days 6, 7, 8).

Figure 34. Formation of differentiated cell types of mesoderm and ectoderm origin by directed differentiation of pluripotent cells in vitro. Individually seeded aggregates developed from ES cell EBs (control EB) and EBMs (EBM) were scored on days 8, 10 and 12 for the presence of (A) beating cardiocytes (mesoderm-derived) and (B) neurons (ectoderm-derived).

Figure 35. The large molecular weight component of MEDII promotes formation of neurons from EPL cells. Individually seeded aggregates developed from EPL cell EBs cultured in 1, and 4, DMEM; 2 and 5, DMEM + anti-human LIF antibody; 3 and 6, DMEM+ anti-human LIF antibody + 100μg/ml R were assessed on days 7, 8, 9 and 10 for the presence of beating cardiocytes (1, 2, 3) and neurons (4, 5, 6).

Figure 36. Formation of mesoderm by reaggregation of EPL cells formed in suspension. 20 μg total RNA from EBMs (day4) which had been trypsinised to single cells and reaggregated in DMEM (IC+B) +/- 10 ng/ml FGF4, or DMEM + 50% MEDII (MEDII) +/- 10 ng/ml FGF4 was analysed after 2 and 4 days for expression of *brachyury* and *Oct4* by northern blot. Representative aggregates in DMEM and DMEM + 50% MEDII at day 4 are shown.

Figure 37. Nascent mesoderm in EPL cell EBs can be programmed to formation of haemopoietic cells. ES and EPL cell EBs were cultured in methylcellulose supplemented with IL-3 and M-CSF to induce formation of macrophages. The proportion of aggregates containing macrophages was determined on days 12, 15 and 18.

Figure 38. Comparative analysis of methodologies for directed formation of mesoderm (EPL EBs) and ectoderm (EBM) germ layers from pluripotent cells. Seeded aggregates were scored at day 10 for formation of beating cardiocytes and day 12 for formation of neurons.

Table 1. Summary of the gene expression patterns of ES cells and EPL cells compared to gene expression in the ICM and primitive ectoderm of the embryo. RNA was isolated from ES cells and EPL cells grown in the presence of MEDII for 2 days and analysed by Northern blot for the expression of *Oct4*, *uvomorulin*, *Fgf5*, *Rex1*, *AFP*, *H19*, *Evx1* and *Brachyury*. *Gbx2* expression was detected using RNA protection assays. Alkaline phosphatase activity was detected using an enzymatic stain on ES and EPL cell layers. Embryonic expression patterns were determined by Rosner et al., 1990, Sch^ler et al., 1990, Yeom et al., 1991 (*Oct4*); Hahnel et al., 1990 (alkaline phosphatase); Sefton et al., 1992 (*uvomorulin*); Haub and Goldfarb, 1991 Hébert et al., (*Fgf5*); Rogers et al., 1991 (*Rex1*); Bulfone et al., 1993; Chapman et al., 1997 (*Gbx2*); Dziadek and Adamson, 1978 (*AFP*); Poirier et al., 1991 (*H19*); Bastian and Gruss, 1990, Dush and Martin, 1992 (*Evx1*); and Herrmann, 1991 (*Brachyury*).

Table 2. ES cells and reverted EPL cells, but not EPL cells, contribute to the development of chimeric mice when injected into CBA/C57 black host blastocysts. Summary of results from the injection of E14TG2a ES cells, their EPL cell derivatives grown in 50% MEDII for 2 and 4 days (EPL;2 and EPL;4 respectively) and reverted EPL cells, formed by the culture of EPL cells in medium containing mLIF but not MEDII for 6 days (EPL;2^R and EPL;4^R).

Table 3. The effect of purified ECM components on EPL cell stability in culture. ES cells were seeded onto tissue culture plastic pretreated with the ECM components gelatin, laminin, plasma fibronectin, collagen IV and a mix of laminin, plasma fibronectin and collagen IV in DMEM + 50% MEDII. After 5 days cultures were stained for alkaline phosphatase and the percentages of EPL cell colonies with no associated differentiation were determined.

Table 4. Individual 5.5 d.p.c. embryos were seeded into 2 ml collagen IV treated tissue culture wells in either embryo culture medium or embryo culture medium + 50% MEDII. After 5 days explants were fixed in 4% PFA and stained for *Oct4* expression by in situ hybridisation.

Table 5. Summary of the physiochemical properties of the low molecular

weight component of the EPL cell-inducing activity.

Table 6. Amino acid analysis of samples derived by purification (Figure 15) of sfMEDII (active sample) and unconditioned medium (control sample). The sample purified from sfMEDII was analysed with and without hydrolysis.

Table 7. Summary of the EPL cell-inducing activities of amino acids, proline analogues and peptides tested in the presence of R. + = EPL cell-inducing activity (minimum active concentration identified in bold); - = lack of EPL cell formation; * = ES cell death at high concentrations (500 μM AZET, 100 μM 3,4 dehydro-L-proline, 50 μM sarcosine and substance P).

Table 8. Summary of EPL cell formation from ES cells in response to ECM components. ECM components were presented to ES cells in solution (+/- 40 μM L-proline), or as matrices (+/- 40 μM L-proline). Purified bioactivity = large molecular weight component of MEDII purified by protocol 2; MedII = Hep G2 conditioned medium or ECM; + = EPL cell formation; - = no EPL cell formation; +/- = sporadic flattened pluripotent cell colonies. ECM components were used in solution at 10 μg/ml (collagen IV; vitronectin and plasma fibronectin) 3 μg/ml (Laminin) or 2μg/ml (cellular fibronectin and purified bioactivity). Gelatin was used at 0.01% and MEDII at 50%.

EXAMPLE 1

Formation of a primitive ectoderm like cell population. EPL cells, from ES cells in response to biologically derived factors

Materials and Methods

Cell culture conditions

ES cells were cultured in the absence of feeders on tissue-culture grade plastic-ware (Falcon) pre-treated with 0.2% gelatin/PBS for a minimum of 30 minutes. Cells were cultured in Dulbecco's Modified Eagles Medium (Gibco BRL), pH 7.4, containing high glucose and supplemented with 10% foetal calf serum

(FCS; Commonwealth Serum Laboratories), 40 μg/ml gentamycin, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol (β-ME), herein referred to as DMEM, supplemented with 1000 units of LIF under 10% CO_2 in a humidified incubator. Routine tissue culture was performed as described by Smith (1991). E14 ES cells (Hooper et al., 1987) were obtained from Anna Michelska (Murdoch Institute, Melbourne). CCE ES cells (Robertson et al., 1986) were obtained from Richard Harvey (Walter and Eliza Hall Institute, Melbourne). MBL5 (Pease at al., 1990) and D3 (Doetschman et al., 1985) ES cell lines were obtained from Lindsay Williams (Ludwig Institute, Melbourne). CGR8 and E14TG2a (Hooper et al., 1987) ES cells were provided by Austin Smith (Centre for Genome Research, Edinburgh).

LIF was produced from COS-1 (ATCC CRL-1650) cells transfected with a mouse LIF expression plasmid, pDR10, as described by Smith (1991) with the following modifications. COS-1 cells were transfected by electroporation using a Bio-rad Gene Pulsar at 270 Volts and a capacitance of 250 μFD. Transfected cells were plated at $7x10^4$ cells/cm^2 in DMEM, pH 7.4, containing high glucose and supplemented with 10% FCS, 40 μg/ml gentamycin and 1 mM L-glutamine. Medium was collected and assayed for LIF expression as described by Smith (1991). Alternatively, medium was supplemented with 1000 units of recombinant LIF (ESGRO, AMRAD).

Hep G2 cells (Knowles et al., 1980; ATCC HB-8065) were maintained in culture in DMEM and passaged at confluence. To condition medium (MEDII) Hep G2 cells were seeded into DMEM at a density of 5×10^4 cells/cm^2. Medium was collected after 4-5 days, sterilised by filtration through a 0.22 μm membrane and supplemented with 0.1 mM β-ME before use. MEDII was stored at 4°C for 1-2 weeks or at -20°C for up to 6 months without apparent loss of activity.

EPL cells were formed and maintained in media containing 50% MEDII conditioned medium in DMEM with or without the addition of LIF. EPL formation was apparent with the addition of between 10 and 80% MEDII, with optimal culture conditions at 50% MEDII (data not shown).

EPL cells were formed from ES cells and maintained as follows :-

Adherent culture: ES cells were seeded at a density of 1.3×10^4 cells/cm^2 onto tissue-culture grade plastic-ware (Falcon) pre-treated with 0.2% gelatin/PBS for a minimum of 30 minutes in DMEM containing 50% MEDII as described above. EPL cells were maintained in 50% MEDII using routine tissue culture techniques (as described by Smith, 1991).

In suspension aggregates: ES cells were seeded at a density of 1×10^5 cells/cm^2 in suspension culture in bacterial petri dishes in DMEM containing 50% MEDII as described above. The resulting EPL cell aggregates were split 1:2 after 2 days and seeded into fresh DMEM containing 50% MEDII. Control aggregates (EBs) were formed by aggregating ES cells in identical fashion in DMEM without MEDII.

DNA manipulations

All DNA manipulations were carried out using standard protocols (Sambrook et al., 1989). DNA was sequenced using a T_7 Sequenase kit (Pharmacia) as per the manufacturers instructions. Sequencing reactions were resolved on 6% polyacrylamide/urea gels and exposed to X-ray film as described in Sambrook et al., 1989.

Northern Blot and ribonuclease protection analysis

Cytoplasmic RNA was isolated from cultured ES and EPL cell layers using the method of Edwards et al (1985). Total RNA was isolated from EPL cell aggregates that had been washed once in 1 x PBS, pelleted by centrifugation and stored at -20°C. Pellets were resuspended in 1 ml of extraction buffer (50mM NaCl; 50 mM Tris.Cl, pH7.5; 5 mM EDTA, pH 8.0; 0.5% SDS) supplemented with 200μg proteinase K (Merck). Sterile, acid washed glass beads (0.5 mm) were added to just below the meniscus and vortexed vigorously. A further 3 ml of extraction buffer/proteinase K was added and incubated at 37°C for one hour before phenol/chloroform extraction. Nucleic acids were precipitated by the

addition of 400 µl 3M NaAcetate and 4 ml of iso-propanol for 30 minutes at -80°C and collected by centrifugation at 3500 rpm in a benchtop centrifuge. Nucleic acids were resuspended in 400 µl DNase I buffer (50 mM Tris.Cl, pH 8.0; 1 mM EDTA, pH 8.0; 10 mM $MgCl_2$; 0.1 mM DTT), 20 units of RNase free DNase I (Boehringer Mannheim) and incubated for one hour at 37°C. RNA was precipitated by the addition of 40 µl 3M NaAcetate and 1 ml ethanol and collected by centrifugation.

Northern Blot analysis was performed as described in Thomas at al., 1995. DNA probes were prepared from DNA fragments using a Gigaprime labelling kit (BresaGen) or Megaprime kit (Amersham). DNA fragments were isolated form the following plasmids. An *H19* cDNA fragment was excised from LC10-8 (Poirier et al., 1991) as a 778 bp fragment with *Pvu*II. A 462 bp *Stu*I cDNA fragment of *Oct4* (residues 491-593) in Bluescript KS+ was obtained from Dr. H. Schöler (Schöler et al., 1990). A 484 bp fragment was released by *XhoI/Hind*III digestion and used for probe generation. *A Brachyury* specific probe was excised from pSK75 (Herrman, 1991) as a 1600 bp *Eco*RI fragment. An *uvomorulin* probe was excised from F20A in pUC8 (Ringwald et al., 1987) as a 620 bp *Eco*RI fragment. A 700 bp *Pst*1/*Bam*H1 *Evx1* cDNA fragment was excised from pAB11 (Dush and Martin, 1992). *Fgf5* was obtained as a full length coding region clone in Bluescript (Hébert et al., 1990), from which an 800 bp *Eco*RI/*Bam*H1 cDNA fragment was isolated and used as a probe. An *AFP* probe was generated from a 400 bp *Eco*RI fragment encoding the first 350 bp of the mouse *AFP* cDNA cloned into in pBluescript KS II+ (courtesy of Dr. R. Krumlauf, NIMR, London). An 848 bp *Rex1* fragment, cloned into pCR™II (pRex1, obtained from Dr. N. Clarke, Department of Genetics, Cambridge University, U.K.) was excised by *Eco*RI digestion. A *mGAP* probe was synthesised by labelling a whole plasmid containing 300 bp of *mGAP* cDNA sequence (Rathjen at al., 1990). The 737 bp, 232 bp and 458 bp ddPCR fragments of *L17*, *K7* and *Psc1* respectively were released from Bluescript II KS + by *Eco*R1 digest and used as probes.

RNase protection assay methodologies and antisense probes for the detection of *Gbx2* and *mGAP* were as described in Chapman et al. (1997). An

antisense riboprobe for the detection of *Oct4* was transcribed using T3 RNA polymerase from the *Oct4* containing plasmid described above which had been linearised with *NciI*.

Northern blots and RNase protections were exposed on phosporimager screens (Molecular Dynamics) and developed on a Molecular Dynamics Phosphor Imager machine. Levels of gene expression were quantified using ImageQuant (Molecular Dynamics) software.

In situ hybridisation

In situ hybridisation on cell layers, cell aggregates and whole mouse embryos was performed using the method of Rosen and Beddington (1993) with the following modification. Cell layers and cell aggregates were pre-hybridised, hybridised and washed at 60°C, and embryos were pre-hybridised, hybridised and washed at 65°C (*L17*; *K7*) and 63.5°C (*Psc1*). Cell layers, cell aggregates and embryos were blocked with 10% heat inactivated FCS and antibodies were added in TBST/1% FCS. The antibodies were not pre-adsorbed in the case of in situ hybridisation of cell layers or cell aggregates. Antibodies were preadsorbed with embryo powder (Harlow and Lane, 1988) prior to incubation with mouse embryos.

Outbred Swiss embryos were taken from time-mated Swiss female mice on the days specified. 0.5 days post coitum was designated as noon on the day of plugging. Mated female mice were killed by cervical dislocation or CO_2 asphyxiation, and uteri were kept in DMEM supplemented with 10 mM HEPES, pH 7.4 at 37°C until dissection of embryos. Embryos were removed, using standard dissection techniques, in PBS. Reichart's membrane was removed and the embryos transferred directly to 4% paraformaldehyde (PFA) in PBS fixative.

Anti-sense *Oct4* probes were synthesised by T3 RNA polymerase as run-off transcripts from bluescript containing a 462 bp *StuI* *Oct4* cDNA fragment (Schöler et al., 1990), linearised with *Hind*III. Sense transcripts, used as controls, were obtained from the same plasmid linearised with XhoI and transcribed with T7 RNA polymerase. Sense and anti-sense *Fgf5* transcripts were generated from a

plasmid clone (Hébert et al., 1990) containing the full-length *Fgf5* coding sequence which had been linearised with either *EcoR*I or *Bam*H1 and transcribed with T7 or T3 RNA polymerase respectively. Sense and antisense probes for *L17*, *Psc1* and *K7* were generated from plasmid clones containing 737 bp, 458 bp and 232 bp fragments respectively. Antisense transcripts of *L17, Psc1* and *K7* were produced by *XhoI* digestion and transcription with T3 RNA polymerase (*L17*, *Psc1*), and *HindIII* digestion with transcription with T7 RNA polymerase (*K7*). Sense probes were generated by *BamH1* digestion of clones and transcription using T7 RNA polymerase for *L17* and *Psc1* and T3 RNA polymerase for *K7*. Antisense *Brachyury* probes were synthesised from pSK75 (Herrman, 1991) linearised with *BamHI* and transcribed with T7 polymerase. Sense probes were generated from the same plasmid linearised with *HindIII* and transcribed with T3 polymerase.

Alkaline Phosphatase staining

Alkaline phosphatase was visualised using the diagnostic ~~kit 86-R (Sigma).~~ The kit was used according to the manufacturer's specifications with the following modification; cell layers were fixed in 4.5 mM citric acid, 2.25 mM sodium citrate, 3 mM sodium chloride, 65% methanol and 4% para-formaldehyde prior to washing and staining.

Histological analysis

Embryoid bodies were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight before embedding in paraffin wax and sectioning as described in Hogan et al., 1994. Sections were stained with haemotoxylin:eosin.

DDPCR

Identification of transcripts differentially expressed between ES and EPL cells by differential display PCR was performed as described in Schulz (1997) (*Psc1*) and Liang and Pardee (1992) (*L17*, *K7*). Primers were designed to

incorporate *Eco*RI restriction sites and cDNA products were cloned into *Eco*RI digested pBluescript II KS +.

Results

MEDII effects the transition of ES to EPL cells in adherent culture

When grown in medium supplemented with recombinant LIF (LIF) and in the absence of a feeder cell layer (Figure 1A) ES cells grow as a homogeneous population with greater than 95% of the colonies displaying a distinctive domed colony morphology. To assay for factors capable of inducing ES cell differentiation, ES cells were seeded and cultured in the presence of mLIF and conditioned medium derived from mammalian cell lines. After 5 days the cells were assessed for divergence from the ES cell colony morphology.

When cultured in the presence of 10-80% medium conditioned by Hep G2 cells (MEDII) on tissue-culture grade plastic-ware (Falcon) pre-treated with 0.2% gelatin/PBS ES cells gave rise to a morphologically distinct population of cells, which we have termed early primitive ectoderm-like, or EPL, cells (Figure 1B). The formation of EPL cells was specific for MEDII and was not seen in response to any of thirteen other conditioned media assayed. In contrast to the characteristic ES cell colony morphology, EPL cells grew as monolayer colonies in which individual cells, containing nuclei with one or more prominent nucleoli, were easily discernible. Morphologically, EPL cells resembled P19 EC cells (McBurney and Rogers, 1982; Rudnicki and McBurney, 1987), which are considered similar to cells of the primitive ectoderm. The culture of ES cells in the presence of' 50% MEDII resulted in a relatively homogeneous cell population in which greater than 95% of the colonies were of the EPL cell colony morphology and in which no residual ES cell colonies could be detected (Figure 1D). This occurred in the presence or absence of added LIF (Figure 1D). Within the EPL cell populations sporadic colonies of differentiated cells, similar to those seen in ES cell cultures, were detected (Figure 1D). The level of differentiated colonies was 4-fold higher in EPL cell cultures formed in the absence of added LIF (Figure 1D), suggesting a possible role for LIF in EPL stability. The formation of a relatively uniform cell

population from ES cells contrasted with the variety of differentiated cell types produced when ES cells differentiate spontaneously in response to LIF withdrawal (Figure 1C,D). However, within spontaneously differentiated ES cell cultures a small proportion of EPL-like colonies were seen (Figure 1D), suggesting that EPL cells are a normal derivative of ES cells. EPL cell morphology could be maintained with extended culture of greater than 40 passages, or 100 days (data not shown) and was dependent on the continued presence of MEDII in the culture medium. The withdrawal of MEDII and LIF resulted in the generation of an array of differentiated cell types (data not shown) similar to those arising from spontaneous ES cell differentiation (Figure 1C).

The transition of ES cells to EPL cells, in response to MEDII, was demonstrated for a number of independently derived ES cell lines including MBL5 D3, CCE, E14 and CGR8 (data not shown). The appearance of EPL cells generated from each ES cell line was comparable.

EPL cells are pluripotent

The sporadic generation of differentiated cells in culture (Figure 1D) and the spontaneous differentiation of EPL cells following withdrawal of MEDII suggested that these cells retained a capacity for further differentiation.

The pluripotent cells of the early mouse embryo and germ line, and ES cells in culture, are characterised by expression of the homeobox gene *Oct4* and by alkaline phosphatase activity. *Oct4* expression is restricted to the pluripotent cells of the early mouse embryo and is down regulated on differentiation of pluripotent cells both in vivo and in vitro. EPL cells were formed by culturing ES cells, in the presence of MEDII or MEDII + LIF, for 2, 4, 6 and 16 days, with passaging every 2 days. Northern analysis of RNA from these populations showed expression of *Oct4* at levels equivalent to the levels seen in ES cells (Figure 2Ai) and suggested that the formation of EPL cells is not equivalent to the process of spontaneous differentiation (Figure 2Aii). In situ hybridisation of ES cells and EPL cell monolayers with an *Oct4* specific anti-sense RNA probe showed *Oct4* expression uniformly distributed across the EPL cells, and not restricted to sub-populations of

cells within the culture (Figure 2B,C). Sporadic differentiated cells within the population did not express *Oct4* (data not shown). Analysis of alkaline phosphatase activity also showed uniform expression by EPL cells (Figure 2D,E), with down regulation of activity in the differentiated cells (data not shown). The uniform distribution of these pluripotent cell markers in EPL cell cultures demonstrated the homogeneity of EPL cell population.

EPL cell formation is accompanied by establishment of primitive ectoderm-like gene expression

The expression of pluripotent cell marker genes by EPL cells suggested that these cells could be equivalent to a pluripotent cell population of the early embryo, or possibly a differentiated cell lineage in which *Oct4* expression was not fully down-regulated. The pluripotent cell populations of the embryo can be discriminated from differentiated cell lineages by morphological and developmental criteria and by the temporal and spatial expression of marker genes. The embryonic equivalent of EPL cells was investigated by analysis of the expression of marker genes that identify the pluripotent cell populations of the embryo, and the differentiated cells of the extra-embryonic lineages and gastrulating embryo.

Three genes, *Fgf5*, *Rex1* and *Gbx2*, have been reported to be differentially transcribed between cells of the ICM and primitive ectoderm (summarised in Table 1). *Fgf5* expression is up regulated on the formation of primitive ectoderm from the ICM, whereas both *Rex1* and *Gbx2* expression can be detected in the ICM but not in the primitive ectoderm by 6.5 d.p.c.. The expression of *Fgf5* and *Rex1* in ES and EPL cells was assessed by Northern blot (Figure 2A; Table 1). *Gbx2* expression was analysed by RNase protection assay (Figure 2F; Table 1). *Fgf5* expression, which was barely detectable in ES cells, was elevated 50 fold in EPL cells grown for 2 days in MEDII. *Fgf5* expression increased in EPL cells with time in culture such that maximal expression, representing a 340 fold induction of *Fgf5*, was reached by day 6 and persisted for at least 16 days in culture. *Rex1* was expressed at high levels by ES cells, but this expression was down regulated 50% within 2 days of EPL cell formation (Figure 2A; Table 1). *Rex1* expression was

reduced further in EPL cells cultured for longer periods of time such that EPL cells grown in MEDII for 6 days showed a 5 fold reduction in *Rex1* expression compared to ES cells. *Gbx2* expression was high in ES cells and maintained with 2 days of culture in MEDII, but was reduced with further culture in MEDII to undetectable levels by day 6. The changes in expression of *Fgf5*, *Rex1* and *Gbx2* were delayed when the transition of ES to EPL cells was carried out in MEDII+LIF when compared to EPL cells formed in MEDII (Figure 2A;2F), suggesting that LIF retarded the ES to EPL cell transition. The persistence of EPL cell morphology and high levels of *Fgf5* and *Oct4* expression (Figure 2A) demonstrated the ability of MEDII to maintain EPL cells as a stable cell population and was clearly distinct from the gene expression changes observed during spontaneous differentiation of ES cells (Figure 2Aii).

EPL cell monolayers, cultured for 4 days in MEDII, were probed for *Fgf5* expression using DIG-labelled anti-sense *Fgf5* transcripts. Equivalent levels of *Fgf5* specific staining were seen in all EPL cells within the culture (Figure 2G) confirming both the uniformity of the ES to EPL cell transition and the homogeneity of EPL cell populations.

The expression of *uvomorulin*, a cadherin expressed by the pluripotent cells of the ICM and the primitive ectoderm but down-regulated in cells of the primordial germ cell lineage, was assessed in ES and EPL cell cultures by northern blot analysis. *Uvomorulin* expression was maintained in EPL cells at levels equivalent to or greater than those observed in ES cells (Figure 2H) suggesting that these cells did not represent an in vitro equivalent of primordial germ cells.

Consistent with the expression pattern of pluripotent cell markers EPL cells did not express detectable levels of marker genes specific for the extra-embryonic lineages of the early embryo, (*H19*, *AFP*) the primitive streak (*Evx1*) or nascent mesoderm (*Brachyury*) (Table 1; data not shown).

The morphological transition of ES to EPL cells was found to be accompanied by differential regulation of marker genes that discriminate pluripotent cell populations in vivo. Although gene expression in ES cells was

equivalent to their origin from the pluripotent cells of the pre-implantation embryo, EPL cells expressed a repertoire of marker genes which is shared by only one cell population of the early embryo, the primitive ectoderm (Table 1).

The expression of novel pluripotent cell marker genes supports the relationship between EPL cells and early primitive ectoderm

The transition of ES and EPL cells appears to mimic the gene expression changes associated with the ICM to primitive ectoderm transition. A screen was carried out to identify genes that are differentially expressed between these cell populations. RNA isolated from ES cells and EPL cells, cultured in MEDII+LIF for 2, 4, 6 and 8 days, was analysed using differential display polymerase chain reaction (ddPCR). cDNA fragments expressed differentially between ES and EPL cells were purified, cloned and sequenced.

Three novel partial cDNAs, *L17*, *Psc1* and *K7*, were identified (Figure 3). Northern blot expression analysis of ES and EPL cell RNA, prepared as outlined above, indicated that *L17* was expressed highly in ES cells but down regulated rapidly with EPL formation and maintenance in culture (Figure 4A). *Psc1* was expressed in ES cells and down regulated in EPL cells more gradually than *L17* (Figure 4B). *K7* was expressed at low levels in ES cells but up regulated on extended EPL cell culture (days 4, 6, and 8; Figure 4C).

In vivo expression of these genes in pre- and peri-implantation mouse embryos was analysed by wholemount in situ hybridisation of mouse embryos from 3.5 d.p.c. to 5.5 d.p.c., which encompasses the ICM to primitive ectoderm transition (Figure 5). Expression of all three novel genes was confined to pluripotent cells at these stages in vivo.

L17 was expressed highly in the ICM at 3.5 d.p.c. and in the pluripotent cells of the ICM/epiblast at 4.5 d.p.c. (Figure 5A). *L17* expression was down regulated as the ICM/epiblast began to proliferate (day 4.75), and was not detectable following pro-amniotic cavity formation.

Psc1 was expressed in the inner cell mass and at 3.5 d.p.c. and in the pluripotent cells of the ICM/epiblast at 4.5 d.p.c. (Figure 5B). Unlike *L17*, *Psc1* expression persisted in the proliferating epiblast bud (4.75 - 5.0 d.p.c.) and was down regulated after the initiation of pro-amniotic cavity formation after 5.0 d.p.c.

K7 expression was not detected in the pluripotent cells of the ICM/epiblast prior to and including 4.5 d.p.c. (Figure 5C). *K7* expression was detected in the pluripotent cells following pro-amniotic cavity formation at 5.25 d.p.c. but was down regulated by 5.5 d.p.c. as the pluripotent cells reorganised to form the columnar epithelial sheet of characteristic of primitive ectoderm.

The strict restriction of *L17*, *Psc1* and *K7* expression to embryonic pluripotent cells in vivo supports the identification of EPL cells as pluripotent. Differential expression of these genes between the ICM/epiblast and primitive ectoderm populations of the embryo was consistent with the identification of EPL cells as distinct from ES cell and more closely related to cells of the early primitive ectoderm.

A role for gp130 signalling in EPL cell maintenance

The increased level of differentiated cell colonies seen in EPL cell cultures without added mLIF when compared to cultures with added mLIF (Figure 1C,D; Figure 6), suggested a possible role for LIF, or other cytokines which signal through gp130, in the maintenance of EPL cells. Antibodies to mouse gp130 (RX-19; Koshimizu et al., 1996), which neutralise the activity of mLIF, hOSM, hIL-6 and hIL-11, but not hLIF, on myeloid leukemic M1 cells and ES cells (Lake, 1996), and to hLIF (R&D Systems), which neutralises hLIF activity, were used to assess the role of gp130 signalling in EPL cell maintenance.

While EPL cell formation was observed in the presence of anti-gp130 (10 μg/ml) or anti-hLIF (10 μg/ml) antibodies, the addition of anti-hLIF antibodies resulted in marked destabilisation of EPL cells compared to cells cultured in MEDII alone (Figure 6). This suggested that the presence of hLIF in MEDII, but not mLIF expressed by the cells, was important for EPL cell maintenance. The addition of

both anti-hLIF antibodies and 1,000 units of mLIF (ESGRO; Amrad) reduced the levels of differentiation to those seen in EPL cell cultures formed in MEDII+LIF, demonstrating that gp130 signalling was able to restore EPL cell stability in culture.

ES cells were seeded into medium containing MEDII, anti-hLIF antibodies (10 µg/ml) and mLIF at concentrations between 10 and 1000 units/ml (ESGRO; Amrad). EPL cell maintenance was achieved with the addition of between 50 and 100 units/ml of mLIF (data not shown), 10 to 20 fold less than the 1000 units/ml required for the maintenance of ES cells.

Formation of EPL cells in suspension culture

Aggregates formed from ES cells in 50% MEDII for 4 days (EBMs) were compared to control aggregates formed from ES cells in the absence of MEDII for 4 days (EBs) at the level of morphology and gene expression. EBs displayed an internal disorganisation in comparison to EBMs, indicated by the random distribution of several internal areas of cell death distributed randomly throughout the EBs in comparison to a uniform central area of cell death in EBMs (Figure 7A). In EBMs this central area of cell death was surrounded by an apparently homogeneous cell layer of uniform thickness. Further, the outer layer of extraembryonic endoderm formed in ES cell EBs could not be discerned microscopically suggesting that EBMs were comprised of a single cell type.

Gene expression analysis was performed by in situ hybridisation and Northern blot analysis on EBs and EBMs developed for 4 days in culture (Figure 7B). Aggregates were analysed for expression of the primitive ectoderm markers *Oct4* and *Fgf5*, and *brachyury*, a gene up regulated on the differentiation of pluripotent cells into nascent mesoderm. The expression of *Oct4* in EBs was patchy, with areas of cells not staining, suggesting that a proportion of the cells in the aggregate had undergone differentiation. By contrast, *Oct4* expression was detected uniformly throughout the cell layer comprising the EBMs, suggesting that these cells remained pluripotent and had not undergone differentiation. This

conclusion was supported by expression of the differentiation specific marker *brachyury* in approximately 10% of the EBs, suggesting that a proportion of the cells in these aggregates had undergone differentiation to nascent mesoderm. In contrast, no *brachyury* expression was detected in the EBMs, indicating that cells within these aggregates had not undergone any differentiation to nascent mesoderm.

The expression of *Fgf5* in aggregates was analysed to assess the formation of EPL cells/primitive ectoderm (Figure 7B). In EBMs *Fgf5* was expressed homogeneously throughout the cell layer, indicating that the pluripotent cells in these aggregates represented a uniform population of pluripotent cells which had undergone the transition to form EPL cells/primitive ectoderm. In contrast, *Fgf5* expression in EBs was heterogeneous.

Northern blot analysis of RNA extracted from EBs and EBMs supported the findings of the in situ analysis (Figure 8). At day 4, EBM gene expression was characterised by high levels of *Oct4* and *Fgf5*, diagnostic for primitive ectoderm. *Brachyury* expression, diagnostic for nascent mesoderm, could not be detected. ES cell EBs expressed a range of genes consistent with the formation of heterogeneous cell types.

The summation of the expression data suggested that in contrast to EBs, in which a variety of cell types ranging from early primitive ectoderm to extraembryonic endoderm to differentiated cells, could be detected, EBMs consisted of a uniform population of cells equivalent to EPL cells and embryonic primitive ectoderm. Control aggregates, developed in 100 units of LIF without MEDII, did not develop as EBMs demonstrating that the alterations seen in pluripotent cell differentiation in the presence of MEDII could not be attributed to the low level of LIF present in MEDII (data not shown).

Summary

The data in this example demonstrate that the conditioned medium, MEDII, contains a biological activity which directs uniform differentiation of ES cells to an

alternative stable pluripotent cell population termed early primitive ectoderm-like or EPL cells. EPL cells were identified as most closely related to cells of the early primitive ectoderm based on gene expression and morphology. The transition from ES to EPL cell could be carried out in adherent culture or in suspension aggregates.

EXAMPLE 2

ES and EPL cells represent distinct, but interchangeable, pluripotent cell states

Materials and Methods

All cells and tissue culture techniques were as described in Example 1 unless otherwise stated. EPL cells were reverted by seeding a single cell suspension of EPL cells into medium containing 1000 units of LIF in the absence of MEDII at a density of 1.3×10^4 cells/cm^2.

Blastocyst injection

ES and EPL cells were introduced to into CBA/C57 F2 blastocysts using standard blastocyst injection technology, as described in Stewart, 1993.

GPI analysis

Glucose Phosphate Isomerase (GPI) analysis of blood and tissues was as described by Bradley, 1987. Blood samples were collected from the tail. The tissue samples analysed were taken from the brain, eye, femur, heart, intestines, kidneys, liver, lung, muscle, stomach, skin, spleen, tongue, thymus and the ovary or testes. Tissue samples were prepared by freeze/thawing and by homogenisation.

Results

EPL cells seeded and cultured in medium containing LIF, but not MEDII,

were observed to adopt an ES cell-like colony morphology, a process we termed reversion. Further, the withdrawal of MEDII, in the presence of LIF, from established EPL cell colonies resulted in the formation of a three dimensional colony structure suggestive of the ES cell phenotype (data not shown). Northern blot analysis was used to examine the expression of *Oct4*, *Fgf5* and *Rex1* in ES, EPL and reverted EPL cells (Figure 9A). EPL cells cultured and passaged in MEDII and MEDII+LIF for 2, 4 and 6 days were reverted by passaging the cells into medium containing LIF alone for 6 days. Reverted EPL cells exhibited low *Fgf5* expression and high *Rex1* expression, comparable to the gene expression profile observed in ES cells and distinct from the expression of high levels of *Fgf5* and low levels of *Rex1* in the parental EPL cells. These data indicated that the phenotypic reversion of EPL cells was accompanied by the establishment of an ES cell gene expression profile. These data also demonstrated a requirement for MEDII for both the establishment and maintenance of EPL cell characteristics.

Clonal EPL cell lines were generated and reverted to ensure that reversion was not a consequence of residual ES cells within EPL cell populations. EPL cells, grown for 4 days in medium containing MEDII but without added LIF, were seeded at limiting dilution in MEDII+LIF to produce clonal EPL cell colonies. Two clones were expanded in MEDII+LIF over 3 weeks before seeding into medium containing mLIF alone or MEDII+LIF. The resulting cultures were assessed for the presence of ES cells, EPL cells and differentiated colonies (Figure 9B). A high proportion of cells from both lines formed alkaline phosphatase positive colonies with ES cell morphology in cultures seeded and maintained in LIF alone which were not seen in the cultures maintained in MEDII+LIF, indicating efficient reversion of the clonal lines.

Reverted EPL cells, but not EPL cells, contribute to chimeric mice following blastocyst injection.

Pluripotent cells from the pre- and post-implantation embryo differ by the ability of the former but not the latter to contribute to embryo development following blastocyst injection. ES cells retain the ability to contribute to all embryonic and adult tissues. E14TG2a ES cells and their EPL cell derivatives

were tested for their ability to contribute to chimeric mice when injected into CBA/C57 F2 blastocysts. The contribution of ES cell and EPL cell derivatives to mouse offspring was assessed by coat colour contribution and GPI analysis of blood and tissues. E14TG2a ES cells contributed to the development of 44% of injected blastocysts (Table 2). In contrast, E14TG2a derived EPL cells, grown in MEDII without added LIF for 2 and 4 days such that EPL cell gene expression was established, did not contribute to chimera formation as assessed by coat colour of the 33 and 50 live born pups respectively (Table 2). GPI analysis of blood samples taken from 20 of these mice also failed to detect any contribution from the EPL cells. Analysis of 15 tissue samples taken from two mice also failed to detect any EPL cell contribution. This could not be explained by adverse effects of EPL cells on the viability of injected blastocysts as the percentage of live born pups was comparable from blastocysts injected with ES cells and EPL cells (Table 2).

The EPL cells used in the preceding blastocyst injection experiments were reverted by culture in media containing LIF, but not MEDII, for 6 days (2^R and 4^R). These cells contributed to embryonic development in 36% (2^R) and 63% (4^R) of blastocysts injected (Table 2). GPI analysis of blood and tissue samples taken from 10 and 2, respectively, of the chimeric mice generated using reverted EPL cells established that these cells were able to contribute to mesodermal, endodermal and ectodermal derived cell lineages of the adult mouse (data not shown). These data indicated that the ES to EPL cell transition resulted in cells of differing developmental capabilities, reflected in the ability of ES cells, but not EPL cells, to contribute to development when introduced into a host blastocyst. The ability of reverted EPL cells to contribute to chimera development suggests that this loss in chimera forming ability by EPL cells can not be attributed to a loss of pluripotence on EPL cell formation.

Summary

The ability of EPL cells to revert to an ES cell is consistent with the predicted and demonstrated behaviour of other pluripotent cell types, such as the formation of EG cells from primordial germ cells (PGCs) in culture, and supports

the deduction that EPL cells are pluripotent. Further, reversion to an ES cell type is a predicted feature of primitive ectoderm. In combination with the demonstrated inability of EPL cells to contribute progeny to chimaeric mice following blastocyst injection, this supports the identification of EPL cells as distinct from ES cells and most closely related to primitive ectoderm. Finally, reversion demonstrates that EPL cells can be used as a substrate for the generation of ES cells in vitro by dedifferentiation or reversion.

EXAMPLE 3

Isolation of EPL and ES cells from embryonic and in vitro-derived primitive ectoderm

Introduction

Existing procedures and culture conditions have failed to support the maintenance and/or proliferation of pluripotent cells from the primitive ectoderm of any mammalian species. Successful isolation and maintenance of pluripotent cells and cell lines from the primitive ectoderm would provide an alternative methodology for isolation of genetically manipulable pluripotent cells with potential for commercial, medical and agricultural application. In the mouse, such lines have only been isolated from the pluripotent cells of preimplantation embryos (ES cells) or primordial germ cell lineages (EG cells). Efficient isolation of pluripotent cell lines is currently restricted to a limited number of inbred mouse strains such as 129, and has not proven successful in other mammals.

MEDII supports the formation and maintenance of EPL cells which are most closely related to the pluripotent cells of the primitive ectoderm in the mammalian embryo. In this example we discuss the development of methodology which utilises the biological activity of MEDII to isolate and maintain pluripotent cells from primitive ectoderm.

Materials and Methods

Cells and cell culture

Cell culture conditions, for ES and EPL cells are as in Example 1 except where otherwise specified. Cells isolated from primitive ectoderm were cultured in Dulbecco's Modified Eagles Medium (Gibco BRL), pH 7.4, containing high glucose and supplemented with 15% foetal calf serum (FCS; Commonwealth Serum Laboratories), 40 μg/ml gentamycin, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol (β-ME), and 1000 units of LIF (embryo culture medium) under 10% CO_2 in a humidified incubator. Inactivated feeder layers were prepared from either STO cells (ATCC CRL-1503) or primary mouse embryonic fibroblasts (Abbondanzo et al., 1993). Inactivation of feeder cells was achieved by exposing cells to 30 gray of ionising radiation. Feeder cells were seeded at least 6 hours before use at a density of 1×10^5 cells/cm^2 in DMEM onto tissue culture plastic treated with human placental collagen IV (Sigma) as per manufacturerís instructions. Feeder layers were washed once with DMEM before the addition of culture medium and embryonic explants. Pluripotent cells were identified using the pluripotent specific markers alkaline phosphatase and *Oct4* and in situ analysis techniques as described in Example 1.

Embryoid bodies (EBs) were formed as described in Example 1.

Source of embryonic cells

CBA/C57 F2 embryos were taken from time-mated CBA/C57 F1 female mice on the days specified. 0.5 days post coitum was designated as noon on the day of plugging. Mated female mice were killed by cervical dislocation or $C0_2$ asphyxiation and uteri were kept in DMEM supplemented with 10 mM HEPES, pH 7.4 at 37°C until dissection of embryos. Embryos were removed, using standard dissection techniques, into DMEM supplemented with 10 mM HEPES, pH 7.4. Removal of extraembryonic endoderm was achieved mechanically by pipetting embryos through a pasteur pipette that had been pulled in a flame to a bore diameter slightly smaller than the embryo.

Results

Isolation of pluripotent cells from primitive ectoderm formed in vitro

Embryoid bodies (EBs), formed by the aggregation of ES cells in suspension culture, follow a pathway of differentiation equivalent to early mouse embryogenesis with the ordered appearance of extraembryonic and differentiated cell types. The appearance of these cells can be monitored by alterations in gene expression. By day 4 of EB development the pluripotent cell population within the embryoid body is comprised largely or entirely of primitive ectoderm, as determined by expression of the primitive ectoderm markers *Fgf5* and *Oct4*, and down regulation of the ES/ICM cell marker *Rex1* (Figure 10A). Pluripotent cells in EBs are largely differentiated by day 8/9 of culture.

Individual EBs, developed for 4 to 8 days in culture, were treated with 0.5 mM EGTA in 1xPBS for 5 minutes before being trypsinised to a single cell suspension. Each single cell suspension was divided equally between two 2 ml tissue culture wells containing either DMEM + LIF or DMEM + 50% MEDII + LIF. After 5 days, cultures were stained for alkaline phosphatase activity to identify pluripotent cell colonies. The presence of MEDII in the medium resulted in the isolation of numerous pluripotent cell colonies (Figure 10B), demonstrating that this factor allowed efficient isolation and maintenance of pluripotent EPL cells from pluripotent cells within the EBs. These EPL cells originated from primitive ectoderm which was shown to be the only pluripotent cell population within the EBs at these stages by the expression of *Fgf5* and *Rex1* (Figure 10A), and by the failure of medium supplemented with LIF alone to support pluripotent cell proliferation and maintenance (Figure 10B). LIF has been shown to be sufficient for the maintenance of pluripotent ICM or ES cells. Pluripotent cells could be isolated from EB derived primitive ectoderm in either the presence or absence of LIF, but was dependent on the presence of MEDII.

These data indicate that MEDII can support the maintenance and proliferation of pluripotent cells from the primitive ectoderm of EBs. The pluripotent cells isolated from the primitive ectoderm were equivalent to EPL cells

in morphology and gene expression, and could be reverted to ES cells when cultured in the presence of LIF and the absence of MEDII (data not shown; Example 2). Pluripotent cells isolated from the primitive ectoderm of EBs in this way and reverted to ES cells have been shown to contribute to chimaeric mice when introduced into host blastocysts.

Isolation and maintenance of pluripotent cells from primitive ectoderm of mouse embryos

The goal of this investigation was the isolation and maintenance of pluripotent cells from the primitive ectoderm of a non-129 strain of mice. Successful isolation and maintenance of pluripotent cells from this strain, which does not normally give rise to ES cells at high frequency, would provide a generic technology that could be extended to other mammals.

Primitive ectoderm from 5.5 d.p.c. embryos is a preferred substrate for pluripotent cell isolation

Whole embryos dissected from time mated female mice at 5.5, 6.5 and 7.5 (d.p.c) were dissected free of Reichart's membrane and placed individually into 2 ml tissue culture wells, pre-treated with 0.1% gelatin for 30 minutes, in 1 ml embryo culture medium + 50% MEDII. After 3 days in culture at 37°C, 10% CO_2, embryonic explants were stained for alkaline phosphatase, and the presence and abundance of alkaline phosphatase positive cells was assessed. The number of embryo explants containing alkaline phosphatase positive cells was greatest in explants of day 5.5 d.p.c. embryos (varying between 25% and 40%) and decreased significantly with increasing age of the embryo to less than 5% in explants of day 7.5 d.p.c. embryos. The abundance of alkaline phosphatase positive cells in explants of 5.5 d.p.c embryos was variable, but most explants comprised 20% or greater alkaline phosphatase positive cells. 5.5 d.p.c. embryos were used as the source of primitive ectoderm in subsequent experiments.

Successful isolation and maintenance of pluripotent cells from the primitive ectoderm has been achieved from embryos collected between 5.25 and 5.5 d.p.c..

Collection of embryos after this window of opportunity, even by 1-2 hours, can compromise the procedure. Explants from these embryos quickly become differentiated and no primitive ectoderm cell layers can be identified morphologically. The isolation of cells from embryos earlier than 5.25 d.p.c. has not been attempted.

Purified extracellular matrix (ECM) components stabilise EPL cells in culture

The effect of alternative extracellular matrix activities on the stability of EPL/primitive ectoderm cells in culture was tested by seeding ES cells at low density (2.5×10^2 cells/cm^2) in DMEM + LIF or DMEM + 50% MEDII onto tissue culture plastic which had been pretreated with gelatin (Example 1), plasma fibronectin, laminin, collagen IV (all obtained from Sigma and used as per the manufacturers specifications) and a combination of plasma fibronectin, laminin and collagen IV. The selected purified ECM components have been shown to be present in the extracellular matrices of the early embryo. Cells were cultured for 5 days and stained for alkaline phosphatase. Colonies were classified on the presence or absence of differentiated (alkaline phosphatase negative) cell types within the colony and the presence of differentiated cells was used as a measure of pluripotent cell stability (Table 3). Of the conditions tested gelatin was the least favourable for EPL cell stability, with 78% of EPL cell colonies containing at least some differentiated cells. The purified matrix components all resulted in more stable EPL cell cultures when compared to gelatin, with collagen IV and the matrix mix containing collagen IV resulting in 59% and 62.5% undifferentiated EPL cell colonies respectively. ES cells cultured in the absence of MEDII were stable on all the tested matrices.

Tissue culture plastic pretreated with collagen IV was used for the culture of pluripotent cells from primitive ectoderm in subsequent experiments.

The biological activity within MEDII is required for the proliferation and maintenance of embryonic primitive ectoderm

The role of MEDII in primitive ectoderm maintenance in culture was

assessed by placing dissected 5.5 d.p.c. embryos individually into 2 ml tissue culture wells, pretreated with collagen IV, in embryo culture medium or embryo culture medium + 50% MEDII and maintained at 37°C, 10% CO_2. After 5 days in culture explants were fixed with 4% PFA and the presence of pluripotent *Oct4* positive cells was assessed by in situ hybridisation (Figure 11; Table 4). Embryos cultured in the presence of LIF became rapidly disorganised and necrotic, clearly lacked a primitive ectoderm cell layer, and did not contain any *Oct4* positive cells after 5 days. By contrast, many of the embryos maintained in the presence of 50% MEDII + LIF retained their embryonic organisation including a clearly identifiable primitive ectoderm cell layer which stained strongly and uniformly for the pluripotent cell marker *Oct4* (Figure 11B). 54% of surviving explants cultured in the presence of 50% MEDII + LIF contained *Oct4* positive cells indicating that MEDII promotes the maintenance of primitive ectoderm-derived pluripotent cells in culture.

Comparative analysis of multiple experiments indicated that fresh MEDII, as opposed to MEDII that had been frozen, was more effective for maintenance in vitro of pluripotent cells derived from embryonic primitive ectoderm.

Other parameters pertaining to successful isolation and maintenance of embryo-derived primitive ectoderm in vitro

The visceral endoderm is recognised as a source of inductive signals involved in the differentiation of pluripotent cells during gastrulation. Removal of visceral endoderm from embryonic explants was performed to eliminate this source of differentiation inducing signals and promote pluripotent cell stability in culture. Although the effect of this step has not been quantified, successful isolation and maintenance of pluripotent cells from the embryo has not been achieved from explants comprising primitive ectoderm and extraembryonic endoderm.

Further, primitive ectoderm explants which failed to adhere to the tissue culture substratum during the initial stages of in vitro culture were shown to contain proliferating primitive ectoderm with fewer contaminating differentiated

cells. These explants were a preferred source of pluripotent cells for further culture. Suspension culture of primitive ectoderm explants was achieved the addition to 2 ml tissue culture wells of 0.5 ml 0.5% agarose in DMEM medium base (Low melting point agarose, Sigma), which was washed 3 x 30 minutes in embryo culture medium before being preequilibrated against embryo culture medium containing 50% MEDII.

Isolation and maintenance of pluripotent cells from the primitive ectoderm of 5.5 d.p.c. embryos (Figure 12)

Embryos were dissected from the uteri of time mated female mice between 10 and 11 a.m. on the morning of the sixth day of gestation (approximately 5.4 d.p.c.). Each embryo was dissected to remove Reichart's membrane, the extraplacental cone and the visceral endoderm layer, resulting in a cup shaped structure comprising primitive ectoderm in the absence of contaminating cell types (Figure 12B). All tissues were removed from the embryos mechanically. Primitive ectoderm explants were placed individually into 2 ml wells plugged with agarose and cultured in 1 ml of embryo culture medium + 50% MEDII. Embryos were cultured at 37°C in a 10% CO_2 humidified incubator.

On the second or third day of culture surviving explants were removed from suspension, redissected to remove any differentiation arising in culture and placed back into 2 ml wells prepared as described above.

On day 5 of culture surviving explants (approximately 25% of explants seeded on day 0), which usually contained identifiable epithelial sheets of *Oct4* positive cells equivalent in many respects to EPL cells (Figure 12B), were removed from suspension, redissected again and plated onto tissue culture plastic pretreated with collagen IV in embryo culture medium + 50% MEDII. Explants were maintained in embryo culture medium + 50% MEDII for a further 2 days. Explants at this stage were usually comprised of convoluted epithelial sheets (Figure 12B). Explants were then cultured in embryo culture medium in the presence of LIF and absence of MEDII. This resulted in flattening of the sheets and adoption of an ES cell like appearance (Figure 12B). Analysis of these cells

by in situ hybridisation showed them to be pluripotent as assessed by *Oct4* (Figure 12B) and alkaline phosphatase expression.

On day 9 or 10 of culture the explants were disaggregated into single cells and small clumps of cells by trypsinisation and seeded into 2 ml wells pretreated with collagen IV or preseeded with inactivated feeder cells. The cells derived by this method were equivalent in appearance to ES cells (Figure 12B), expressed the pluripotent cell markers alkaline phosphatase and *Oct4* (Figure 13), retained the ability to differentiate spontaneously and proliferated in culture for at least four weeks. Pluripotent cells were isolated from approximately 10% of embryos, an efficiency comparable with the isolation of ES cells from the ICM of 129 mouse blastocyst stage embryos by standard techniques.

Summary

The work described in this example provides the first demonstration that pluripotent cells from embryonic primitive ectoderm can be isolated and maintained in culture as pluripotent cells analogous to EPL and ES cells for extended periods. Culture and passage of these cells was dependent on the biological activity contained within MEDII. Pluripotent cells obtained in this manner are likely to be amenable to genetic manipulation and represent a resource with potential commercial, medical and agricultural application. The demonstration that pluripotent EPL and ES cells can be isolated from the primitive ectoderm of mouse species that are refractory to ES and EG cell isolation by other methodologies potentially provides an opportunity for the isolation of pluripotent cell lines from other mammalian and avian species.

The demonstration that cells equivalent to EPL cells can be isolated and maintained from embryonic primitive ectoderm cultured in the presence of the biological activity contained within MEDII provides further support for the proposed similarity between EPL cells and embryonic primitive ectoderm.

EXAMPLE 4

Purification of components of the biologically active factor from serum free conditioned medium

Material and Methods

All cells and tissue culture techniques were as described in Example 1 unless otherwise stated. Purification of EPL cell-inducing factors utilised standard FPLC and HPLC chromatographic techniques with activity assessed via morphology based assay for ES cell to EPL cell conversion. Amino acids, proline analogues and peptides were purchased from Sigma.

Serum free MEDII (sfMEDII) was used as a source of the biologically active factor in all purification protocols. SfMEDII was shown to cause the ES to EPL cell transition in a manner analogous to MEDII (Example 1; data not shown). To produce sfMEDII, Hep G2 cells were seeded at a density of 5 x 10^4 cells/cm^2 and cultured for three days. Cells were washed twice with 1 x PBS and once with serum free medium (DMEM containing high glucose but without phenol red, supplemented with 1 mM L-glutamine, 0.1 mM β-ME, 1 x ITSS supplement (Boehringer Mannheim), 10 mM HEPES, pH 7.4 and 110 mg/L sodium pyruvate) for 2 hours. Fresh serum free medium was added at a ratio of 0.23 ml/cm^2 and the cells were cultured for a further 3-4 days. SfMEDII was collected, sterilised and stored as for MEDII (Example 1). Large-scale production of sfMEDII was carried out in corrugated roller bottles (Falcon).

Cell assays for EPL cell-inducing activity

Morphology based assays to test the EPL cell-inducing activity of purified fractions were performed with D3 ES cells. Assays were carried out in 2 ml wells (Falcon) in a total volume of 1 ml/well. ES cells (2.5×10^2 cells/cm^2) were cultured in the presence of semi-purified fractions and scored microscopically for EPL cell morphology after 4 days in culture, and macroscopically after 5 days following staining for alkaline phosphatase activity.

Extracellular matrix preparations were formed as follows. Hep G2 cells were grown to confluence under conditions described in Example 1. Cells were incubated in 0.5% Sodium Azide/0.1mM PMSF in PBS for 6-18 hours at 37°C or room temperature to kill and detach cells, or cultured in 0.5mM EGTA for 15 minutes at room temperature to detach cells. Cells and debris were removed by washing with PBS. Formation of matrices from semi-purified or purified proteins was achieved by drying 1-2 µg of protein onto a 2 ml tissue culture well. ES cells were seeded onto matrices at a concentration of $2.5x10^2$ cells/cm^2 in DMEM supplemented with 1000 units/ml LIF and 10 µg/ml L-proline. Cell morphology was assayed microscopically on day 4 and following staining for alkaline phosphatase activity on day 5.

The presence of proline in semi-purified samples was detected directly by thin layer chromatography of samples on 0.2mm silica gel 60 F_{254} plates (Merck) in 80% propanol followed by staining with ninhydin.

Protease digestion

250mg Collagen IV (Sigma) was digested with 3 FALGPA units of collagenase type IV (Sigma) in 50 mM Tris.Cl pH 7.4, 100 mM $CaC1_2$ buffer in a final volume of 10 ml and incubated overnight at 37°C with continuous mixing. Digested collagen fragments were separated from enzyme and undigested protein by ultrafiltration through an Amicon Diaflo YM3 membrane using a 400ml ultrafiltration cell (Amicon) at 4°C under nitrogen pressure. The eluate obtained from ultrafiltration was passed through a 0.22 µm filter and 1.5 ml aliquots were lyophilised and resuspended in 200 µl water. 4x50 µl of the resuspended filtrate was applied to a Superdex peptide gel filtration column equilibrated in water and connected to a SMART micropurification system (Pharmacia). 25 µl fractions were collected and assayed directly for EPL cell-inducing activity in the presence of R.

For trypsin digestion of the large molecular weight component of the EPL cell-inducing activity, 80 µl of R, at 3.6 mg/ml, was digested for 1 hour at 25°C with 100 units of trypsin (Difco) in 20 mM Tris.Cl pH 8.5, 20 mM $CaCl_2$. The reaction

was stopped by the addition of 100 µM PMSF. The sample was concentrated on a Centricon-10 column (Amicon) and assayed for the large molecular weight component of the EPL cell-inducing activity.

Protein analysis by reducing PAGE and western blot

Protein samples were obtained directly from conditioned medium, or chromatographic fractions were desalted on a Centricon-10 column (Amicon) and made to the original volume with 20 mM Tris.Cl pH 8.5. Samples were electrophoresed on 10% reducing SDS polyacrylamide gels as described by Laemmli, 1970 and proteins were visualised by silver staining using the method of Heukeshoven and Dernick (1985).

For western blotting, gels were washed for 30 minutes in western transfer buffer (39 mM glycine, 48 mM Tris.Cl, 20% methanol, 0.037% SDS) and electroblotted on a Mini-Protean II apparatus (BioRad) in this buffer to nitrocellulose at 400 mAmps constant voltage for 3 hours. Filters were blocked overnight in PBT (0.1% Triton X-100, 1X PBS), 5% skim milk powder. Monoclonal antibody 3E2 (Sigma), specific for the EDA region of cellular human fibronectin, was diluted 1/1000 in 1% skim milk powder, PBT and incubated at room temperature for 2 hours, before incubation with the filter for 2 hours at room temperature. Filters were washed 3 times in PBT at room temperature, then incubated for 2 hours at room temperature in goat anti-mouse alkaline phosphatase antibody (Dako) diluted 1/10000 in 1% skim milk, PBT. A 20 minute wash in western buffer 1 (100 mM Tris.Cl pH 7.4, 100 mM NaCl) was followed by 2 x 20 minute washes in western buffer 2 (Tris.Cl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The reaction was developed in western substrate mix (10ml western buffer 2, 40ul NBT (75 mg/ml in 70% DMF), 40ul BCIP (50 mg/ml in 100% DMF)) in the dark and stopped by the addition of 10 ml Western buffer 1, 100 mM EDTA.

Results

Soluble biological factors within MEDII are responsible for the ES to EPL cell transition

SfMEDII was separated into two fractions by ultrafiltration through a 10×10^3 M_r cut-off membrane (Centricon-3 unit; Amicon). Both fractions were assayed for EPL forming activity (Figure 14), defined as the ability to cause the complete conversion of D3 ES cells to alkaline phosphatase positive EPL cells in the presence of mLIF. ES cells seeded into the retained fraction ($>3 \times 10^3$ M_r) at concentrations equivalent to 50% MEDII did not form EPL cells, although there was an increase in the size of the ES cell colonies. ES cells seeded into the eluted fraction ($<3 \times 10^3$ M_r) at concentrations equivalent to 50% MEDII gave rise to an array of colony morphologies including ES, EPL and differentiated cells. The presence of ES cells and the higher proportion of differentiated cells was uncharacteristic of the ES to EPL cell transition, demonstrating that the eluted material alone was unable to induce EPL cell formation. Seeding of ES cells into medium containing both the retained and eluted fractions at concentrations equivalent to 50% MEDII resulted in uniform EPL cell formation, equivalent to that seen for sfMEDII. These data indicated that two separable biological factors were required for the conversion of ES to EPL cells.

Large scale preparation of R and E fractions from sfMEDII

The starting material for purification and analysis of bioactive factors from MEDII was derived by ultrafiltration of sfMEDII over an Amicon Diaflo YM3 membrane using a 400ml ultrafiltration cell (Amicon) at 4°C under nitrogen pressure. The retained fraction (R), $>3 \times 10^3$ M_r, was used immediately or aliquoted and stored at -20°C. The eluted fraction (E), $<3 \times 10^3$ M_r, was used immediately or stored at 4°C.

Cell assay for detection of the low molecular weight component of the EPL cell-inducing activity

The low molecular weight component of the EPL cell-inducing activity was assayed as described above except that 1ml culture medium was supplemented with 20 µl (50-100 µg protein) of R which had been passed over a PD10 column (Pharmacia) to remove low molecular weight contaminants.

Physiochemical properties of the low molecular weight component

The physiochemical properties of the low molecular weight component of the EPL cell-inducing activity were deduced from treatment and assay of E (Table 5). Activity was maintained following acid treatment at pH 2.0, repeated freeze-thawing, boiling for 1 hour, and reduction with 50 mM DTT at room temperature. Although the active component was soluble in water, acetonitrile, methanol and propanol, making it amenable to HPLC purification, it did not bind to ion exchange or reverse phase HPLC columns using standard techniques.

Purification of the low molecular weight component of the EPL cell-inducing activity

220 ml of E was applied to a Sephadex G10 column (1100 ml bed vol, 110x113 mm) equilibrated in water. Elution was with water at room temperature at a flow rate of 35 ml/minute. Fractions of 45 ml were collected and a 1 ml aliquot of each fraction was lyophilised. Lyophilised fractions were resuspended in 100 µl of water and 25 µl was assayed for EPL cell-inducing activity. Activity was detected in fractions 6-10, 19-25.2 minutes after injection (Figure 15A).

Fractions 7 to 9 were pooled, lyophilised and resuspended in 1 ml of 30:70 methanol:acetonitrile. Samples were centrifuged at 14,000rpm for 10 minutes to remove precipitates and applied to a 10 mm Waters radial pak normal phase silica column (8 mm I.D.) attached to a Waters 510 HPLC machine. The column was washed with 30:70 methanol: acetonitrile at a flow rate of 0.2 ml/minute for 15min

before the material was eluted with a 20min linear gradient against water using a flow rate of 0.5 ml/minute. Eluted material was detected with a Waters 490E programmable multiwavelength detector set at 215 nm. 1 ml fractions were collected, lyophilised, resuspended in 50 µl DMEM and assayed for EPL cell inducing activity which eluted from the column at 70% water / 30% (30:70 methanol:acetonitrile) (Figure 15B).

The fractions of highest activity from normal phase chromatography, between 32 and 35 minutes, were lyophilised, resuspended in 50 µl water and 10 µl was applied to a Superdex peptide gel filtration column (Pharmacia) connected to a SMART micropurification system (Pharmacia) and equilibrated in water at room temperature. The column was eluted with water at a flow rate of 25 µl/minute (Figure 15C) and 25 µl samples were collected. This was repeated 5 times to obtain adequate sample for analysis. Individual samples were assayed directly for bioactivity which was detected in fractions eluting approximately 71.04 to 74.04 minutes after injection in a single peak or several closely eluting peaks (ie fractions 8, 9 and 10). The predicted molecular weight of the active fractions was <700D according to the elution volume.

Characterisation of the purified low molecular weight component.

Fraction 9 from the Superdex peptide gel filtration column was lyophilised, derivatised with FMOC and OPA and amino acid analysis was conducted with and without hydrolysis using a Hewlett-Packard Amino-Quant II analyser. Results were compared with a control sample of non-conditioned medium subjected to an identical purification (Table 6). The amino acid alanine and the imino acid proline were present in abundance compared to the control in both hydrolysed and unhydrolysed samples. This indicates that these amino acids were present within the purified sample as free amino acids and not a peptide.

L-proline (3×10^{-4}M) and L-alanine (3.9×10^{-4}M) were assayed for EPL cell-inducing activity. L-proline effected conversion of ES cells to EPL cells in the presence of R in a manner indistinguishable from the low molecular weight

component of sfMEDII. This included the alterations in morphology, and *Fgf5* and *Rex1* expression reported in Example 1 (data not shown). L-alanine had no effect on ES cells in the presence or absence of R.

Addition of 10^{-4}M L-proline to ES cells in the absence of R resulted in a heterogeneous population containing some cells/colonies morphologically similar to EPL cells. The addition of R was necessary for complete and homogeneous EPL cell formation.

The minimal active molar concentration of proline was found to be approximately 40μM. The optimal concentration in biological assays was found to be 100μM.

The effect of proline analogues and proline-containing peptides on ES and EPL cells

Proline analogues reported to have proline-like bioactivity in other cell systems and proline-containing peptides were tested over a range of concentrations to assess their ability to form EPL cells from ES cells in the presence or absence of R. Results are presented in Table 7.

The proline analogues D-proline, trans-4-hydroxy-L-proline, pyrrolidine, N-acetyl-L-proline, N-t-BOC-proline and L-pipecholic acid (PCA), had no observable effects on ES cell growth or differentiation in the presence or absence of R. Sarcosine, L-azetidine-2-carboxylic acid (AZET) and 3,4 dehydro-L-proline inhibited ES cell growth at lower concentrations and caused ES cell death at higher concentrations without inducing EPL cell formation in the presence or absence of R. These data point to a structural requirement for proline activity in EPL cell formation that is distinct from that described for other proline bioactivities.

The proline containing peptides ala-pro, gly-pro, pro-OH-pro, ala-pro-gly, gly-pro-ala, gly-pro-arg-pro, val-ala-pro-gly, gly-pro-gly-gly, substance P free acid (arg-pro-lys-pro-gln-gln-phe-phe-gly-leu-met-OH), substance P fragment 1-4 (arg-

pro-lys-pro) and Bradykinin (arg-pro-pro-gly-phe-ser-pro-phe-arg; data not shown), in combination with R, were able to cause conversion of ES to EPL cells at similar molar concentrations to L-proline (Table 7). The peptides pro-ala, pro-gly and gly-pro-OH-pro in the presence of R were also able to cause the conversion of ES to EPL cells, but at a molar concentration approximately 6 times higher than that of proline (Table 7). The lack of correlation between the specific activities and the molar amount of proline in these peptides provides further evidence for a structural requirement for the action of this factor, and points to the possibility that it interacts with a receptor.

EPL cells were also formed when ES cells were cultured in the presence of R and partially purified collagen IV hydrolysate, resulting from collagenase digestion of collagen IV. Free L-proline could not be detected within the partially purified collagen IV hydrolysate by TLC. The bioactivity of collagen IV hydrolysate presumably reflects the presence of repeating Gly-Pro-X motifs in collagen IV proteins and provides a possible source of this bioactivity in vivo by breakdown of ECM components.

The carboxy-terminus of substance P (fragment 5-11; gln-gln-phe-phe-gly-leu-met-OH; data not shown), the integrin binding peptide RGD, the neurokinin A and B receptor antagonists (neurokinin A and senktide) and the amino acids L-alanine and L-lysine had no observable effect on ES cells in the presence or absence of R, and used at concentrations equal to or greater than the bioactive peptides described above.

At concentrations above 50 μM, the tachykinin substance P induced apoptosis of ES cells. Compared to substance P free acid and substance P fragment 1-4, substance P has a markedly increased affinity for the NK-1 receptor.

These results indicated that L-proline and proline-containing peptides have the bioactivity associated with the low molecular weight component of sfMEDII. The identification of multiple factors with similar but distinct specific activities

points to structural requirement for bioactivity, suggests the involvement of receptor interaction, and allows prediction of additional peptides with the low molecular component of the EPL cell-inducing activity.

Purification of the large molecular weight component of the EPL cell-inducing activity

Cell assay for detection of the large molecular weight component of the EPL cell-inducing activity

The large molecular weight component of the EPL cell-inducing activity was assayed as described previously except that 1ml culture medium was supplemented with 500 µl of E (made to 10% FCS) or 40 µM L-proline.

Unless otherwise stated, fractions were prepared for inclusion in the assay as follows. The protein concentration of semi-purified fractions of R from chromatographic purification steps was estimated using a BioRad Protein Assay kit (BioRad # 500-0001). Aliquots of each fraction containing 100 µg of protein were concentrated on centricon-10 units (Amicon), washed with 2 ml of PBS and reconcentrated. Each fraction was assayed for the large molecular weight component of the EPL cell-inducing activity at a range of concentrations, from 0.1 µg to 10 µg of protein.

Physiochemical properties of the large molecular weight component

The physiochemical properties of the low molecular weight component of the EPL cell-inducing activity were deduced from treatment and assay of R, prepared as above except that an Amicon Diaflo YM10 membrane was used for ultrafiltration. R was passed over a PD10 column (Pharmacia) to remove low molecular weight contaminants and used in assays at approximately 50-100 µg/ml. Treatment of R by incubation at >56°C for 1 hour, or digestion with the protease trypsin resulted in the loss of biological activity. The large molecular weight component of the EPL cell-inducing activity was also unstable at pH 5.5 and below, or following treatment with reducing agents such as 100 mM DTT for 4

hours at room temperature. These data indicated that the large molecular weight component of the EPL cell-inducing activity was proteinaceous.

Large molecular weight component bioactivity can be detected in the extracellular matrix (ECM) of cultured Hep G2 cells

ES cells were seeded onto Hep G2 ECM in 2 ml tissue culture wells at low cell density, 2.5×10^2 cells/cm^2, and cultured for 5 days in DMEM + LIF, with or without L-proline. Cultures were stained for alkaline phosphatase activity after 5 days and the cultures were assessed by morphology for EPL cell formation. Matrix from Hep G2 cells was able to act as a source of the large molecular weight component of the EPL cell-inducing activity and effectively induced the formation of EPL cells when used in combination with L-proline (Table 8). When used alone the matrix was unable to induce complete conversion of ES to EPL cells, however a number of the colonies adopted a flattened or spread morphology.

Purification of the large molecular weight component from sfMEDII: Protocol 1

R, prepared from 4 l of sfMEDII as described above, was made to 20 mM Tris.Cl pH 8.5 and passed over a 300 ml Sepharose Q anion exchange column (Pharmacia). Bound proteins were eluted via a step wise gradient of 200 ml 20 mM Tris.Cl pH 8.5, 200 mM NaCl, 200 ml 20 mM Tris.Cl pH 8.5, 400 mM NaCl and 200 ml 20 mM Tris.Cl pH8.5, 1 M NaCl (Figure 16A). 200 ml samples were collected and assayed for their ability to form EPL cells in the presence of 40 μM L-proline. Activity eluted from the column in 400 mM NaCl.

The active fraction from anion exchange chromatography was made to a final concentration of 0.5M ammonium sulphate in 20 mM Tris.Cl pH 8.5. The sample was filtered through a 0.22 μm filter to remove precipitated material before being passed over a 40 ml phenyl Sepharose fast flow hydrophobic interaction column (Pharmacia). Bound protein was eluted from the column in a single step with 50 ml 20 mM Tris.Cl pH8.5 (Figure 16B) and shown by assay to contain the

large molecular weight component of the EPL cell-inducing activity.

The active fraction from hydrophobic interaction chromatography was diluted in 20 mm Tris.Cl pH8.5 to a conductivity below 100 mM NaCl. The sample was passed over a 10 ml Heparin Sepharose CL-6B column (Pharmacia). Bound protein was eluted from the column in a single step with 10 ml 0.5M NaCl, 20 mM Tris.Cl pH 8.5 (Figure 16C) and shown by assay to contain the large molecular weight component of the EPL cell-inducing activity.

The active fraction from Heparin Sepharose affinity chromatography was passed down a Superose 6 gel filtration column (Pharmacia) on the SMART system (Pharmacia) in 20 mM Tris.Cl pH 8.5, 150 mM NaCl (Figure 16D). 50 µl fractions were collected and assayed directly. Fractions containing bioactivity equivalent to the large molecular weight component of sfMEDII eluted as a single peak between 500-1000 kDa. EPL cell formation was only observed in the presence of E or L-proline.

Reducing SDS PAGE revealed the presence of a highly purified protein of approximately 210-260 kDa in samples containing the large molecular weight component of the bioactivity (Figure 16E).

Purification of the large molecular component from sfMEDII: Protocol 2

1 litre of sfMEDII was applied to a 50 ml Heparin Sepharose CL-6B column (Pharmacia) equilibrated in 20 mM Tris.Cl, pH 8.5. The column was washed sequentially with 200ml 20 mM Tris.Cl, pH 8.5, 200 ml 20 mM Tris.Cl, pH 8.5 + 150 mM NaCl, 200 ml 20 mM Tris.Cl, pH 8.5 + 500 mM NaCl and 200 ml 20 mM Tris.Cl, pH 8.5 + 1 M NaCl (Figure 17A). Aliquots of each wash were assayed for biological activity on ES cells. The large molecular weight component of the EPL cell-inducing activity eluted from the column in the 500 mM NaCl fraction.

The active fraction from Heparin Sepharose affinity chromatography was diluted to 150 mM NaCl with 20 mM Tris.Cl, pH 8.5 and applied to a 1 ml Resource Q anion exchange column (Pharmacia) pre-equilibrated with 20 mM

Tris.Cl, pH 8.5 + 150 mM NaCl. Proteins were eluted from the column with a gradient from 150 mM to 500 mM NaCl over 60 minutes, followed by a 5 minute wash in 1 M NaCl at 1ml/min (Figure 17B). Alternate 1.5 ml fractions were assayed for the high molecular weight component of the EPL cell-inducing activity which eluted from this column in 200-300 mM NaCl.

Fractions 10-17 from anion exchange chromatography were pooled and passed down a Superose 6 gel filtration column (Pharmacia) in 20 mM Tris.Cl, pH 8.5 + 150 mM NaCl (Figure 17C). 50µl fractions were collected and assayed directly for the presence of the large molecular weight component of the EPL cell-inducing activity which eluted as a single peak between 500-1000 kDa.

The active fraction was analysed by reducing SDS PAGE and shown to contain a highly purified protein of approximately 210-260 kDa (Figure 17D), equivalent to the protein purified in protocol 1.

Purification of the large molecular component from sfMEDII: Protocol 3

4 litres of sfMEDII was applied to a 25 ml gelatin Sepharose affinity chromatography column (Pharmacia), pre-equilibrated in PBS, at a flow rate of approximately 5 ml/minute. The column was re-equilibrated in PBS and proteins were eluted in a 50 ml volume of 6 M urea in PBS (Figure 18A). The eluate was dialysed against 4 x 4 litre of PBS at 4°C to remove urea. The protein concentration of the eluate was determined using the BioRad protein assay kit and 0.1-10 µg was assayed for bioactivity.

The eluate was analysed by reducing SDS PAGE and shown to contain a highly purified protein of approximately 210-260 kDa (Figure 18B), equivalent to the protein purified in protocols 1 and 2. The yield of purified protein was determined by Bradford analysis to be approximately 1 mg/litre sfMEDII.

Characterisation and identification of the large molecular weight activity

The highly purified active fractions obtained from purification protocols 1-3

were able to induce EPL cell formation in the presence of E or L-proline when used either in solution at 2 μg/ml (Figure 19C) or when pre-coated onto tissue culture plastic (data not shown). This is consistent with previous identification of the protein as a component of the ECM. EPL cell-inducing activity could not be detected in the absence of E or L-proline.

The extra-cellular matrix proteins human laminin (Sigma), bovine vitronectin (Dr. Z. Upton, CRC for Tissue Growth and Repair, Adelaide) collagen IV (Sigma), human plasma fibronectin (Sigma) and human cellular fibronectin (J-P Levesque, IMVS, Adelaide) were tested for the ability to promote the formation of EPL cells from ES cells in tissue culture media in the presence of 40 μM proline and compared to the active fractions (Table 8). Of these, only cellular fibronectin at a concentration of ≥ 1 μg/ml, was able to effect the formation of EPL cells from ES cells when added in solution (Figure 19 A, B). The activity of cellular fibronectin, which is a homodimer of 250 kDa disulphide bonded proteins, was consistent with the size and characteristics of the highly purified bioactive factor from sfMEDII identified by reducing SDS PAGE.

Western blot analysis of the active fractions from Superose 6 gel filtration (Protocol 2), using an antibody specific for cellular fibronectin, confirmed the identity of the 240 kDa purified protein as cellular fibronectin (Figure 20).

Multiple ECM proteins can be used to provide the large molecular weight component of the EPL cell-inducing activity when presented to cells as a matrix

The above mentioned basement membrane components were tested for the ability to promote EPL cell formation when pre-coated onto tissue culture plastic. Morphological induction of EPL cell morphology, in the presence of L-proline, was restricted to cellular and plasma fibronectin and the basement membrane component laminin (Table 8).

Fibronectin and laminin bind to cells via cell surface integrin receptors, with the specificity of ligand binding determined by various heterodimeric combinations

between alpha and beta integrins. These data suggest that a range of ECM components, activating the correct integrin receptor, could induce EPL cell formation when presented to ES cells as an extracellular matrix. However, only cellular fibronectin has been shown to induce formation of EPL cells from ES cells when presented in soluble form.

Summary

Size fractionation of sfMEDII revealed a requirement for at least two biologically active factors in EPL cell formation, a large molecular weight and a low molecular weight component. The low molecular weight component of sfMEDII was identified as L-proline, active at concentrations of 40 μM and above. Analysis of proline analogues and small peptides demonstrated that a number of small, proline containing peptides could substitute for L-proline in the formation of EPL cells.

Chromatographic purification by a variety of protocols identified the large molecular weight component of sfMEDII as cellular fibronectin. In the presence of L-proline the large molecular weight component or cellular fibronectin was able to effect the formation of EPL cells both in solution and as a matrix. The ability of several extracellular matrix components to induce formation of EPL cells when presented to ES cells in matrix-associated but not soluble form and in combination with L-proline suggested that the biological activity of cellular fibronectin was mediated by integrins at the cell surface, most probably an alpha 1:beta 5 heterodimer.

Taken together the data presented in this example identify factors that can induce the formation of EPL cells from ES cells when presented in soluble or matrix associated form, and provide sufficient information for the prediction of factors with similar bioactivities, acting via similar receptor/molecular interactions.

EXAMPLE 5

Biologically active components of the EPL cell-inducing activity can be

isolated from divergent sources and species

Materials and Methods

Preparation of primary cell lines

Primary liver cells were prepared using procedures based on procedures described by Giger and Myer (1981), with minor variations. Mice were sacrificed and immediately dissected to expose the liver. Each liver was perfused through the portal vein with 10 ml PBS, followed by 10 ml 0.05% collagenase in Hanks buffered salt solution (HBSS; 0.8g KCl, 0.12g KH_2PO_4, 16g NaCl, 0.1g Na_2HPO_4, 2g glucose, 4 ml 1% phenol red, .035g $NaHCO_3$ in 2 litres). The perfused liver was removed, macerated in 10 ml/liver of 0.05% collagenase/HBSS and incubated at 37°C for 30 minutes. Liver suspensions were regularly agitated by pipetting to aid liver disaggregation. Cells were washed twice in HBSS (10 ml) to remove collagenase, and contaminating eyrthrocytes were lysed in Sassa solution (6.95g NH_4Cl, 2.058g Tris-base, 1g $KHCO_3$/ litre). Following two washes in Williamís E medium (1 ml/liver, Gibco BRL) the cells were seeded into gelatin treated tissue culture flasks in DMEM and incubated at 37°C, 5% CO_2.

Primary avian hepatocytes were prepared from 17-18 day chick embryos. In brief, chick embryos were removed from the egg, decapitated and dissected to expose the heart and liver. The liver was perfused, via cannulation of the heart, with 10 ml of 0.9% NaCl containing 2 mM EDTA to remove blood cells, followed by 4 ml of 0.05% collagenase in HBSS. Livers were removed, pooled in HBSS and transferred into fresh 0.05% collagenase/HBBS when all livers had been perfused and collected. Pooled livers were macerated with fine tipped scissors and incubated for 30 minutes with gentle shaking every 5 minutes and gentle pipetting using a 10 ml pipette every 10 minutes to aid cell disaggregation. Collagenase was removed by washing with HBSS (2 ml/liver). Contaminating erythrocytes were lysed in Sassa solution. Following two washes with Williams E (2 ml/liver) to remove cell debris and haemoglobin from lysed erythrocytes, the hepatocytes were resuspended in Williams E medium (1ml/liver) and the yield

determined. Typically 2 x 10^7 cells/liver were obtained and plated out in a $175cm^2$ tissue culture flask and cultured in 20 ml DMEM at 37°C/5% CO_2.

Preparation of conditioned media

Primary mouse and chicken hepatocytes were cultured for 4 days in DMEM before collection of conditioned medium.

The mouse hepatocellular carcinoma cell lines Hepa-1c1c 7 (ATCC CRL-2026) and Hep 3B (ATCC HB-8064), and the P19 embryonal carcinoma derived cell lines END2 (visceral endoderm like, Mummery et al., 1985) and PYS2 (parietal endoderm like, Lehman et al., 1974), were maintained in DMEM. Conditioned medium was collected after cell lines had been cultured for 4 days in non-gelatinised tissue culture flasks at 37°C, 5% CO_2.

Conditioned medium was processed as described for MEDII in Example 1. Conditioned medium was assayed for biological activity as described in Examples 1 and 4.

Visualisation of bioactive components

The presence of proline in the active fraction of END-2 medium was detected by thin layer chromatography as described in Example 4. Cellular fibronectin was detected by reducing SDS PAGE as described in Example 4.

Results

Conditioned medium from primary mammalian and avian hepatocytes, liver-derived cell lines (Hepa-1c1c 7 and Hep 3B) and embryonic endoderm like cell lines (END-2 and PYS-2), were assayed for the ability to form EPL cells from ES cells.

Conditioned medium from the primary hepatocytes, both mouse and chicken, was able to effect the transition of ES to EPL cells in culture (Figure

21A). This was only true of medium taken from seeded primary liver cells. Passaging resulted in loss of hepatocytes, which were overgrown by cells of fibroblastic appearance, and loss of EPL cell-inducing biological activity. No difference in biological activity was detected in conditioned medium from primary embryonic (chick) and adult (mouse) hepatocytes and MEDII.

Medium conditioned by the cultured cell lines Hepa-1c1c 7, Hep 3B, END-2 and PYS-2 did not induce the formation of EPL cells from ES cells in culture. However, several of these cell lines could be shown to express one component of the biological activity. Conditioned medium from Hepa-1c1c 7 cells, when used in combination with L-proline, effected the transition of ES to EPL cells (Figure 21B), suggesting that these cells express the large molecular weight component of the EPL cell-inducing activity. This was confirmed by reducing SDS PAGE analysis of Hepa-1c1c 7 conditioned medium which showed the presence of a protein with equivalent mobility to cellular fibronectin (Figure 21C). Conditioned medium from Hep 3B cells was unable to effect the ES to EPL cell transition, alone or in combination with components of the biological activity purified from MEDII. These cells, and several other liver-derived cell lines did not express detectable levels of cellular fibronectin (Figure 21C), indicating that expression of the large molecular weight component of the EPL cell-inducing activity is not ubiquitous among liver derived cell lines.

Conditioned medium from END-2 cells induced formation of EPL cells from ES cells when used in conjunction with cellular fibronectin (Figure 21B), suggesting that these cells secrete L-proline or a functional analogue but not cellular fibronectin (Figure 21C). Semipurification of the conditioned medium by normal phase chromatography (Example 4) and analysis by thin layer chromatography demonstrated the presence of L-proline in END-2 conditioned medium (data not shown).

Summary

Biological activities equivalent to that found in MEDII, and capable of

inducing the transformation of ES cells to EPL cells, have been identified in conditioned media from cells of divergent species, including mammals and birds, and in tissues from both the embryo and adult. This demonstrates that the activity has been conserved across these species during evolution and indicates the fundamental importance of this activity in embryonic development. Further, individual components of the EPL cell-inducing activity are expressed by cells derived from diverse lineages.

EXAMPLE 6

Alternative differentiation of ES and EPL cells in vitro

Materials and Methods

Cell culture and in vitro differentiation assays

All cells and tissue culture techniques were as described in Example 1 unless otherwise stated. All EPL cells used for differentiation assays were formed from ES cells cultured in the presence of MEDII for 2 days. The presence or absence of additional LIF is specified for each example.

The KSF4 ES cell line, which constitutively expresses nuclear localised LacZ (Berger et al., 1995; obtained from Patrick Tam, CMIR, New South Wales, Australia), was maintained on inactivated primary mouse embryonic fibroblast feeder layers (Abbondanzo et al., 1993) in DMEM (high glucose) supplemented with 40 μg/ml gentamycin, 20% foetal calf serum (FCS), 0.1 mM β-mercaptoethanol, l mM L-glutamine and 1000 units LIF as before. Prior to the formation of EPL cells, KSF4 cells were cultured on gelatin treated tissue culture plastic, in the absence of a feeder layer, for two passages.

For the majority of experiments detailed here, embryoid bodies (EBs) were formed in medium without the addition of MEDII or LIF (DMEM) using the partial trypsinisation method (Robertson, 1987). Alternatively, EBs were formed from a single cell suspension plated at $1x10^5$ cells/ml in bacteriological dishes in ES

DMEM, or suspended in a hanging drop of medium (50 µl) at 1.6×10^4 on the inside surface of a petri dish lid. Cell aggregates formed in suspended drops were transferred to bacteriological dishes for further culture after 48 hours. EBs were maintained with regular replenishing of medium. Mixed cell EBs were formed from a single suspension as previously described, in which the two cell populations were mixed at the specified ratio.

Results consistent with those presented herein were obtained using EPL cells derived from a variety of ES cell lines including, D3 (Doetschman et al., 1985), MBL5 (Pease et al., 1990), KSF-4 (Berger et al., 1995) and E14 (Hooper et al., 1987), and using alternative methods of EB formation, including single cell suspension, hanging drop and partial trypsinisation. Furthermore the differentiation potential reported for EPL cells was specifically associated with these cells as it could not be recapitulated by ES cells which had been spontaneously differentiated by culture in the absence of LIF for equivalent time periods (data not shown).

Retinoic acid (RA) differentiation of pluripotent cell aggregates was carried out by plating ES or EPL cells in bacteriological dishes, at a density of 1×10^5 cells/ml, in DMEM supplemented with 1 µM RA. After 48 hours, aggregates were transferred to DMEM and maintained for 2 days, then seeded individually into 2 ml tissue culture wells. The presence of neurons was assessed by microscopic examination two days after seeding. Neuron identity was confirmed by positive staining with the neurofilament 200 antibody N-4142 (Sigma).

Cytokine/Growth Factor Assays

Assays for the effect of cytokines/growth factors were carried out on ES cells and EPL cells seeded at low density (75 cell/cm^2) in 4 well multidishes (Nunc) in 0.8 ml of DMEM + LIF or DMEM + 50% MEDII + LIF respectively. Cytokines/growth factors were added at specified concentrations and the assays were stained for alkaline phosphatase activity after 5 days of culture as described in Example 1.

Gene expression analysis

RNA was isolated from ES and EPL cells using the method of Edwards et al (1985). RNA was isolated from EBs and RA-treated aggregates by the method of Chomczynski and Sacchi (1987).

Northern blot analysis and wholemount in situ hybridisation were carried out as described in Example 1. Riboprobes were synthesised as described by Kreig and Melton (1987).

Probe fragments used for northern blot analysis and/or in situ hybridisations were as detailed in Example 1 with the addition of; *AFP*, linearisation of a plasmid containing a 400 bp *EcoRI* fragment encoding the first 350 bp of mouse *AFP* cDNA in pBluescript KS II+ (obtained from Dr. R. Krumlauf, NIMR, London) with HindIII followed by transcription with T3 polymerase; *SPARC*, 570 bp *EcoRI* fragment derived from 13G43 (Mason et al., 1986); *Goosecoid*, linearisation of a plasmid containing 909 bp *goosecoid* genomic DNA (Blum et al. 1992) with HindIII and transcription with T3 polymerase; *Nkx2.5*, linearisation of plasmid containing 1.6 kb *Nkx2.5* cDNA (Lints at al., 1993) with HindIII and transcription with T3 polymerase.

Histological Analysis

EBs were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight and processed and sectioned as described in Example 1. Sections were stained with toluidine blue for 10 seconds, cleared in Histoclear (National Diagnostics) and coverslipped using a xylene-based mounting medium. EBs subjected to in situ hybridisation were fixed in 4% PFA overnight, washed several times with PBS, 0.1% Tween-20, treated with 100% methanol for 5 minutes and then isopropanol for 10 minutes. Bodies were then embedded and sectioned as described in Example 1.

To detect β-galactosidase activity EBs were fixed in 0.2% gluteraldehyde in PBS for 15 minutes on ice, washed 3x15 minutes with detergent rinse (0.1M

phosphate buffer (pH 7.3), 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-450) and stained in detergent rinse containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-gal for 2 hours at 37°C. EBs were then washed 3x with detergent rinse and transferred to 70% EtOH. For sectioning, EBs were dehydrated to 100% ethanol and processed as described in Example 1.

Immunological detection

AFP expression in sectioned EBs was carried out essentially as described by Dziadek and Adamson (1978) with the following modifications. Prior to antibody incubations, endogenous peroxidase activity was blocked by incubation of the section in 3% H_2O_2 in methanol for 30 minutes. Sections were incubated with a 1/200 dilution of rabbit anti-AFP (ICN) for 2 hrs, washed several times with PBS then incubated with 1/200 dilution of HRP-conjugated anti-rabbit IgG (Silenus).

Formation of beating cardiocytes from ES and EPL cells

Beating cardiocyte formation was assessed in individual EBs, formed from either ES or EPL cells, plated onto pre-equilibrated agarose plugs (1% agarose in DMEM medium base equilibrated against DMEM for 3 hours at 37°C, 10% CO_2 in 2 ml tissue culture wells) in DMEM. The presence or absence of beating muscle was assessed at days 4, 6, 8, 10 and 12 by microscopic examination. Alternatively, beating muscle formation was assessed in individual EBs seeded into 2 ml wells at day 6 of development and scored microscopically at days 7, 8, 10, 12, 14 and 16. Seeded aggregates were also scored for the presence of terminally differentiated neurons on days 8, 10 and 12.

Results

Embryoid bodies, formed by aggregation of pluripotent cells in suspension, follow a pathway of differentiation reminiscent of early embryogenesis (Example 1) and provide an opportunity for broad assessment of pluripotent cell differentiation into both embryonic and extraembryonic cell types. The term EB is used herein to

describe pluripotent cell aggregates cultured in DMEM.

Preliminary characterisation of EPL and ES embryoid body formation

Clear morphological differences were apparent when EBs were formed from either ES cells or EPL cells. ES cell EBs comprised a homogeneous population of round, relatively smooth and ordered aggregates (Figure 22A). EBs formed from EPL cells also formed a homogeneous population, however the bodies appeared irregular and disorganised (Figure 22B). Sectioning and staining indicated that while ES cell EBs comprised a relatively uniform population of cells characterised by a compacted round morphology (Figure 22C), reminiscent of undifferentiated pluripotent cells, internal cells in EPL EBs were loosely packed and heterogeneous, possibly reflecting differentiation (Figure 22D). This observation prompted a detailed comparative investigation of ES and EPL cell differentiation.

Accelerated pluripotent cell differentiation in EPL cell embryoid bodies

The progression from ICM to primitive ectoderm to germ layer formation can be monitored via alterations in gene expression both in vivo and during embryoid body differentiation in vitro. *Rex1* is expressed by ICM and ES cells, and is down regulated in EPL cells, the primitive ectoderm at 6.0 d.p.c. and during ES cell differentiation in vitro. *Fgf5* is not expressed by ICM or ES cells, but is expressed in EPL cells and the primitive ectoderm prior to and during gastrulation, and up regulated transiently during ES cell differentiation in vitro. *Oct4* is expressed by all pluripotent cell populations of the embryo, EPL cells and ES cells, and is down regulated upon differentiation of these cells in vivo and in.

Although the expression of *Rex1* was found to be down regulated in both ES and EPL cell embryoid bodies, the kinetics of *Rex1* regulation differed between the two cell populations. Down regulation to a barely detectable, basal level was observed by day 1 in EPL cell embryoid bodies (Figure 23). In contrast, a similar level of *Rex1* expression was not observed in ES cell embryoid bodies until day 2, with intermediate levels of expression observed on day 1. Similarly,

the kinetics of *Fgf5* regulation differed between embryoid bodies derived from ES or EPL cells. *Fgf5*, already expressed in EPL cells, was up regulated rapidly from day 1 in EPL cell embryoid bodies to a peak on day 2/3, followed by a marked decrease in expression on day 4. Up regulation of *Fgf5* expression in ES cell embryoid bodies was not observed until day 3. While expression levels in these bodies increased further at day 4, they remained below *Fgf5* levels in EPL cell bodies at days 2 and 3. *Oct4* expression decreased with time in both ES and EPL cell embryoid bodies but was down regulated more rapidly in EPL cell embryoid bodies, with a 4 fold decrease seen between day 3 and 4 (Figure 23). This decrease in *Oct4* expression followed the highest levels of *Fgf5* expression and presumably reflected differentiation of pluripotent cells within the bodies.

The changes in *Rex1*, *Fgf5* and *Oct4* expression observed in both ES and EPL embryoid body development suggested that differentiation reflected the events of normal embryogenesis and proceeded through the formation of late stage primitive ectoderm. Further, the rapid increase in *Fgf5* expression and the earlier onset of differentiation, as detected by the loss of *Oct4* and *Fgf5* expression, indicated that pluripotent cell differentiation within EPL cell embryoid bodies was accelerated compared to ES cell embryoid bodies. These properties are consistent with our previous alignment of ES and EPL cells with pluripotent cell populations occurring prior to 6.0 d.p.c.

The EPL cell embryoid body environment is non-permissive for visceral endoderm formation

By 4.5 d.p.c. pluripotent cells exposed to the blastocoelic cavity have differentiated to form primitive endoderm. The primitive endoderm gives rise to two distinct endodermal cell populations, visceral endoderm, which remains in contact with the epiblast, and parietal endoderm, which migrates away from the pluripotent cells to form a layer of endoderm adjacent to the trophectoderm. While others have analysed extraembryonic endoderm formation at relatively late stages (day 9-12) of embryoid body development, formation of an outer layer of endoderm, containing both visceral and parietal endoderm cells, can be observed in embryoid bodies by day 4 or 5 of development. This timing is coincident with

formation of primitive ectoderm and creation of an inner cavity, events that accompany endoderm specification in the embryo. Analysis of endoderm formation at this stage is therefore more likely to reflect normal embryonic events.

Formation of parietal and visceral endoderm during EPL and ES embryoid body development was analysed by *SPARC* and *AFP* expression respectively. *SPARC* expression followed similar kinetics in ES and EPL cell embryoid bodies, with an approximately 2 fold increase over the first 4 days (Figure 24A). Wholemount in situ hybridisation and sectioning of ES and EPL cell embryoid bodies indicated that *SPARC* expression was confined to an outer endodermal cell layer in both ES and EPL cell embryoid bodies at day 4 (Figure 24B,C), indicative of parietal endoderm formation. *AFP* levels were too low to be detected during these stages by Northern or RNase protection, so wholemount in situ hybridisation of embryoid bodies was used to detect *AFP* expression. At day 3, 1% of ES cell embryoid bodies exhibited discrete patches of *AFP* expressing cells on their surface. This level rose to 52.9 +/- 9.5% of ES cell embryoid bodies by day 4 (Figure 24D). Sectioning confirmed that *AFP* expression was confined to outer cells and was therefore representative of visceral endoderm (Figure 24F). *AFP* expression could not be detected on surface or interior cells of EPL cell embryoid bodies at day 3 or day 4 of embryoid body development (Figure 24E).

Cell mixing experiments were carried out to determine whether the failure of EPL cells to form visceral endoderm reflected an inherent restriction in the developmental potential of these cells or an alteration in the embryoid body environment. The KSF-4 ES cell line constitutively expresses a *LacZ* gene modified to target β-galactosidase protein to the nucleus. Analysis of embryoid bodies formed from KSF-4 ES cells demonstrated formation of visceral endoderm at levels comparable with D3 ES cells (data not shown). After passaging in the absence of mouse embryonic fibroblast feeder cells, KSF-4 ES cells were cultured for 2 days in MEDII to form KSF-4 EPL cells (EPL (LZ+)). Embryoid bodies generated by co-aggregation of a 1:1 ratio of D3 ES cells and EPL (LZ+) cells were assessed for visceral endoderm formation by *AFP* expression at day 4. ES cell embryoid bodies and EPL (LZ+) cell embryoid bodies gave rise to visceral

endoderm at levels consistent with those previously described (65% and 0% of bodies respectively). Embryoid bodies formed from the mixed cell population gave rise to visceral endoderm at levels comparable to ES embryoid bodies (51%). Double staining for β-galactosidase activity and *AFP* expression revealed the presence of both *LacZ+* and *LacZ-* cells within the visceral endoderm (Figure 25). This suggested that the inability of EPL embryoid bodies to give rise to visceral endoderm resulted from a deficiency in the embryoid body environment rather than an inherent developmental restriction of EPL cells, and implies the existence of an inductive signal for visceral endoderm formation.

Differentiation of pluripotent cells in the absence of visceral endoderm

The formation of EBs from EPL cells provides a methodology for the differentiation of pluripotent cells in the absence of visceral endoderm. Visceral endoderm is known to express signals that influence pluripotent cell differentiation in vivo. Differentiation in the absence of this cell type provides an opportunity for specific control of pluripotent cell differentiation by the addition of factors and environmental alteration. This cannot be achieved in ES cell EBs in which pluripotent cell differentiation is at least partly controlled by signals from visceral endoderm which differentiate spontaneously from exterior ES cells.

Accelerated and enhanced mesoderm formation in EPL cell EBs

The formation of early mesoderm within ES and EPL cell EBs was monitored by analysing expression of the early mesodermal markers, *brachyury* and *goosecoid*. Consistent with previous reports, *brachyury* expression was barely detectable on days 0-3 of development in ES cell EBs, but was up regulated on day 4 (Figure 26). *Goosecoid* expression could not be detected in ES cell EBs during the course of this experiment. In contrast, *brachyury* and *goosecoid* expression were up regulated 30 and 6 fold respectively on days 2 and 3 of EPL cell EB development, followed by 9 (*brachyury*) and 6.5 (*goosecoid*) fold decreases in expression on day 4 (Figure 26). In both ES and EPL cell embryoid bodies the expression of mesodermal markers immediately preceded decreases in the expression levels of primitive ectoderm markers *Fgf5* and *Oct4* (Figure 23).

Brachyury expression in ES cell embryoid bodies did not reach the levels seen in EPL cell bodies at day 3/4 even after extended culture.

Wholemount in situ hybridisation was used to detect the extent of *brachyury* expression within the embryoid body populations. *Brachyury* expression was detected in 1% of ES cell EBs at day 3 and 16% of bodies at day 4 (Figure 26B,D). By contrast, 98% and 92% of EPL cell EBs exhibited *brachyury* expression on days 3 and 4 respectively (Figure 26C,E). A similar expression pattern was observed with *goosecoid* (data not shown). *Oct4* expression within ES and EPL cell embryoid bodies exhibited the expected inverse correlation with the onset and extent of *brachyury* expression. *Oct4* expression was relatively uniform throughout control EBs on days 3 and 4 (Figure 26F,H), but patchy within EPL cell EBs where mesoderm differentiation had commenced (Figure 26G,I). The comparison of markers specific for nascent mesoderm suggested that EPL cell embryoid bodies undergo an accelerated differentiation program resulting in the earlier appearance and more extensive formation of mesoderm compared to ES cell embryoid bodies.

Terminal differentiation of nascent mesoderm was monitored by the appearance of beating cardiocytes (Figure 27A). This was first detected in ES cell EBs at day 8 of development (8%) and increased with time, reaching 36% of bodies at day 12. In EPL cell EBs, beating muscle was observed in 14% embryoid bodies by day 6, two days prior to its appearance in ES cell EBs, and increased steadily to 60% by day 10 and 12 of embryoid body development. The proportion of EPL cell EBs containing beating muscle was higher than ES cell EBs at all time points. Consistent with this profile, expression of *Nkx2.5* was induced earlier in EPL cell EBs, by day 6 compared to day 8 in ES cell EBs (Figure 27B). Furthermore, while *Nkx2.5* expression levels increased in both ES cell and EPL cell EBs to day 12, levels in ES cell EBs were approximately 3 fold below those observed in EPL cell EBs throughout this period. The enhanced ability of EPL cells to form cardiac muscle when differentiated as embryoid bodies presumably reflects the accelerated and increased formation of nascent mesoderm.

The formation and differentiation of EPL cells as EBs therefore provides a methodology for efficient programming of pluripotent cell differentiation into mesodermal lineages. Mesodermal progenitors are formed earlier and at much higher levels than in ES cell EBs.

EPL cell embryoid bodies exhibit reduced capacity for neuron formation

Differentiation of EPL cells into ectoderm derived lineages was assessed by the presence of neurons within individual embryoid bodies (Figure 28A), and compared to ES cell EBs. Neurons were not detected in either embryoid body population before day 10. On day 10, 26% of ES cell EBs contained obvious neural networks. This rose to 41% by day 12. Embryoid bodies derived from EPL cells failed to form neurons.

ES cells and P19 embryonal carcinoma (EC) cells form neurons when differentiated as aggregates in the presence of retinoic acid (RA). To examine whether the failure of EPL cell EBs to give rise to neurons resulted from an inherent restriction in neuron differentiation capacity, the ability of EPL cells to differentiate into neurons after aggregation in the presence of RA was assessed. Individual RA-treated ES and EPL cell aggregates were scored for the presence of neurons (Figure 28B) and found to exhibit similar frequencies of neuron formation at 63% and 68%, respectively. This indicated that the absence of neurons within EPL cell EBs did not reflect an inherent restriction in the capacity of EPL cells to form neurons, but an altered embryoid body environment which resulted in reduced neural specification.

In summary, EPL cell EBs formed nascent mesoderm and mesoderm derivatives with high efficiency, but failed to form neurons despite retaining neuron-forming potential. Differentiation in EPL cell EBs may reflect directed formation of the mesoderm germ layer from pluripotent cells cultured under these conditions. This may result from the demonstrated failure of EPL cell EBs to form an outer layer of visceral endoderm which has been demonstrated to play a role in the specification of ectodermal lineages.

Differentiation of EPL cells in response to cytokines/growth factors

Differentiation of ES cells and EPL cells in response to members of the FGF growth factor family

ES and EPL cells were seeded into DMEM + LIF or DMEM + 50% MEDII + LIF respectively at a density of at 75 cells/cm^2. Cells were cultured in the presence of increasing concentrations (0.1-100 ng/ml) of recombinant bovine basic fibroblast growth factor (bFGF; Boehringer Mannheim) for a period of 5 days. Addition of bFGF to ES cells in the presence of LIF had no effect on ES cell morphology, differentiation or proliferation. In contrast, addition of 10 ng/ml or higher concentrations of bFGF induced the differentiation of EPL cells to a characteristic cell type (cell type A; Figure 17A) which did not stain for alkaline phosphatase. Similar results were obtained with other members of the FGF family including human recombinant acidic FGF (R&D Systems; 10 ng/ml), human recombinant kFGF/FGF4 (Sigma). Human recombinant FGF5 (R&D Systems) did not induce ES or EPL cell differentiation at concentrations up to 500 ng/ml

Individual colonies within representative differentiation cultures induced with 10ng/ml bFGF were classed as ES cell, EPL cell, differentiated cell, or mixed EPL/differentiated cell on the basis of morphology and alkaline phosphatase staining (Figure 29B). The proportion of undifferentiated ES cell colonies was similar regardless of the presence of bFGF. The addition of bFGF to EPL cell cultures caused a marked decrease in the level of EPL cell colonies, from 58% to 21%, with a corresponding increase in colonies containing differentiation (42% to 79%), indicating that bFGF was inducing the differentiation of EPL cells but not ES cells in culture.

Activin A induces the differentiation of EPL cells but not ES cells.

ES and EPL cells were seeded at a density of 75 cell/cm^2. Cells were cultured for 5 days in DMEM + LIF or DMEM + 50% MEDII + LIF respectively, to which had been added increasing concentrations of recombinant human activin A (1-200 ng/ml; courtesy of Dr. R. Rogers, Flinders Medical Centre, S.A.). Activin A had no effect on ES cell morphology, differentiation or proliferation at any

concentration tested. Addition of activin A to EPL cells in culture induced their differentiation. After 5 days the majority of colonies in activin A treated wells had a distinctive colony morphology (Figure 29C) characterised by the presence of a fibroblastic cell type which did not stain for alkaline phosphatase activity. This cell type was morphologically distinct from cell type A, formed by differentiation of EPL cells with bFGF, and is referred to as cell type B.

Individual colonies within representative differentiation cultures induced with 150ng/ml activin A were classed as ES cell, EPL cell, differentiated cell, or mixed EPL/differentiated cell on the basis of morphology and alkaline phosphatase staining (Figure 29D). The proportion of undifferentiated ES cell colonies was similar regardless of the presence of activin A. The addition of activin A to EPL cell cultures resulted in efficient cell differentiation as assessed by a marked decrease in the proportion of EPL cell colonies from 52% to 3%, and an increase in the proportion of colonies containing differentiated cells from 48% to 97%.

Reverted EPL cells regain ES cell differentiation capability

EPL cells revert to ES cells when cultured in the absence of MEDII but in the presence of LIF as assessed by morphology, gene expression, cytokine responsiveness and ability to contribute to chimaeras following blastocyst injection (Example 2). ES cells were differentiated to EPL cells by culture in MEDII for 2 days, before passaging into medium containing LIF to form reverted EPL cells (EPL^R). Embryoid bodies were formed from each ES, EPL and EPL^R cell population and their differentiation was compared.

Embryoid bodies derived from EPL^R cells were identical in morphology to ES cell EBs and could be distinguished easily at day 4 from EPL cell EBs (Figure 22; data not shown). Expression of *brachyury* in ES and EPL cell EBs (Figure 30A) was consistent with the profile previously described (Figure 26). *Brachyury* expression in EPL^R cell EBs was similar to that observed for ES cell EBs, with expression detected at low levels on day 4 of development and not at high levels on day 2 and 3 as described for EPL cells. Compared to EPL EBs, which failed to give rise to visceral endoderm, EPL^R EBs gave rise to visceral endoderm at levels

comparable to ES EBs (31% and 46% respectively), as shown by wholemount in situ hybridisation. Microscopic analysis of individually seeded ES, EPL and EPL^{R} EBs (Figure 30B,C) indicated that reversion of EPL to EPL^{R} cells was accompanied by restoration of high levels of neuron formation and reduced levels and later appearance of beating cardiocytes. Reverted EPL cells therefore have a similar differentiation capacity to ES cells both in vitro and in vivo (Example 2).

Summary

Comparative analysis of ES and EPL differentiation confirmed the pluripotent nature of EPL cells, supported the identification of EPL cells as primitive ectoderm-like, and defined ES and EPL cells as developmentally distinct populations on the basis of alternate differentiation capabilities and growth factor responsiveness. In combination with Example 2, this indicates that ES and EPL cells have distinct differentiation capacities both in vitro and in vivo.

The products of ES and EPL cell differentiation within embryoid bodies differ both in the proportion of ectodermal and mesodermal germ layers and derivatives, and in the formation of the extraembryonic lineage visceral endoderm. EPL cell embryoid body formation therefore provides:

- a methodology for the differentiation of pluripotent cells in the absence of visceral endoderm and the inductive signals produced by visceral endoderm. This provides unprecedented opportunity for control of pluripotent cell differentiation including directed lineage specific differentiation.

- a methodology for efficient programming of pluripotent cell differentiation to a mesodermal fate by differentiation to EPL cells followed by formation of embryoid bodies or treatment with mesoderm-inducing growth factors/cytokines.

EXAMPLE 7

Alternative formation of ectoderm and mesoderm germ layers and derivatives by directed differentiation of pluripotent cells in vitro

The use of factors within MEDII has enabled the development of a strategy for the production of a uniform population of pluripotent EPL cells in vitro, either as colonies in adherent culture or as aggregates in suspension, representative of primitive ectoderm. Further, the failure of EPL cell embryoid bodies to form the instructive cell type visceral endoderm, and the responsiveness of EPL cells to inductive signals that do not differentiate ES cells provides an opportunity for direct control of pluripotent cell differentiation.

In this example we demonstrate that the differentiation of pluripotent EPL cells can be controlled in vitro to generate cell types representative of specific embryonic germ layers, notably ectoderm and mesoderm. These germ layer cells can be used for the generation of differentiated derivatives with potential for commercial, medical and agricultural use. Specifically, manipulation of environmental stimuli, such as alteration of the extracellular matrix and/or the addition of exogenous soluble factors, can be used for efficient and programmed formation of alternative germ layers. Further manipulation of the environment will enable the directed formation of individual cell lineages from the germ layers, allowing the production of specified cell types and differentiation intermediates. Examples of this strategy are outlined below.

Materials and Methods

Cell culture conditions

All cells and tissue culture techniques were as described in Examples 1 and 6 unless otherwise stated.

Lineage specific differentiation: formation of neurectoderm

For the production of neurectoderm, EBMs (Example 1) were collected on day 4 and resuspended in DMEM + 50% MEDII with or without 20 ng/ml FGF4

(Sigma) in bacterial petri dishes and maintained for a further 3 days.

Cellular aggregates used for the analysis of gene expression by in situ hybridisation were subsequently seeded for 16 hours (day 8) onto tissue culture plates pre-coated with gelatin prior to fixing with 4% PFA. Control aggregates were ES cell EBs formed from ES cells in DMEM in the absence of MEDII. Cellular aggregates used for the analysis of gene expression at timepoints after day 8 by in situ hybridisation were seeded onto gelatinised tissue culture plastic in 50:50 DMEM:F12 (F12, Gibco BRL) + 10% FCS. After 16 hours the medium was changed to 50:50 DMEM:F12 supplemented with ITSS (Boehringer Mannheim), 1 mM L-glutamine and 10-20 ng/ml recombinant human FGF4. Aggregates were maintained in this medium for 1-4 days prior to fixing with 4% PFA.

For functional differentiation assays to assess the ability of neurectoderm formed from ES cells to differentiate into neurons, aggregates on day 7 were seeded individually into 2 ml tissue culture wells and maintained as described above. The formation of neurons was assessed on days 8, 10 and 12.

Lineage specific differentiation: formation of mesoderm

Formation of cells of mesodermal lineage has been achieved using several similar approaches.

From EPL cells generated in adherent culture: Mesoderm was formed from EPL cells formed by adherent culture in the presence of MEDII as described in Example 1. These cells were trypsinised to a single cell suspension and seeded at a density of 1 x 10^5 cells/ml into bacterial plates to form EPL cell EBs as described in Example 6.

From EPL cells formed in suspension (EBMs): EPL cells, formed as cell aggregates (EBMs) in suspension culture, were differentiated into mesoderm by trypsinisation to a single cell suspension and reaggregation in bacterial dishes in DMEM or DMEM + 10 ng/ml FGF4. Cells were seeded at a density of 1 x 10^5

cells/ml.

Macrophage formation from ES and EPL cells

On day 0, ES and EPL cells were trypsinised and seeded in DMEM into bacterial grade petri dishes at a density of $1x10^5$ cell/ml to allow aggregate formation. On day 2, approximately 100 aggregates from each plate were collected and seeded into 1.25ml MC media (0.9% methyl cellulose in Iscoves modified Dulbecco's medium (IMDM), 15% FCS, 50 mg/ml ascorbic acid, and $4.5x10^{-4}$M monothioglycerol (MTG)) supplemented with 400 U/ml IL-3 (courtesy of Dr. T. Gonda, IMVS, Adelaide) and 10 ng/ml recombinant human M-CSF (R&D Systems). On Day 14 and Day 18, 50 colonies of each cell type were scored as containing 5 or more macrophages (positive) or less than 5 macrophages (negative).

Cells formed in MC culture were collected by centrifugation, cytospun, and air dried. Macrophages were identified by morphology when stained with May-Grünwald-Giemsa stain, and by positive staining with the macrophage-specific antibody F4/80 (Austyn and Gordon, 1981).

Gene expression analysis

Gene expression analysis by in situ hybridisation, northern blot and RNase protection were performed as described in Examples 1 and 6. Additional probes were: *Sox1* riboprobes for wholemount in situ hybridisation were generated from plasmid #1022 (obtained from Dr. Robin Lovell-Badge, NIMR, London) linearised with *BamHI* and transcribed with T3 RNA polymerase (anti-sense) or linearised with *HindIII* and transcribed with T7 RNA polymerase (sense). Antisense *Sox1* probes for RNase protections were generated from a plasmid containing a ~450 bp *Xho*1/*EcoR*1 fragment from the *Sox1* cDNA in plasmid #1022 subcloned into pBluescript II KS+. The resulting plasmid was linearised with *Kpn*1 and transcribed with T7 RNA polymerase. *Sox2* probes for use in in situ hybridisation were generated from plasmid #1015 (obtained from Dr. Robin Lovell-Badge, NIMR, London) linearised with *AccI* and transcribed with T3 RNA polymerase

(antisense) or linearised with *NotI* and transcribed with T7 RNA polymerase (sense). An anti-sense *Gbx2* riboprobe for use in in situ hybridisation were generated from pGbx2c7.1 (Chapman et al., 1997) linearised with *AluI* and transcribed with T3 RNA polymerase.

Immunohistochemical Analysis

Seeded cellular aggregates were fixed in 4% paraformaldehyde in PBS and blocked in the appropriate blocking buffer for 30 minutes followed by overnight incubation with primary antibodies in blocking buffer at 4°C. Aggregates were then rinsed with species specific secondary antibodies in blocking buffer for 1 hour. For FITC-conjugated secondary antibodies, propyl gallate 5 mg/ml was added. Slides were examined on a Nikon TE300 microscope using fluorescence.

Nestin: Blocking buffer: 1% goat serum, 1 mg/ml BSA, 1% Triton X 100 in PBS. Primary antibody: Developmental Studies Hybridoma Bank, reference Rat 401, used at a dilution of 1:100. Secondary antibody: FITC conjugated goat anti-mouse IgM (μ-specific: Sigma) used at a concentration of 1:100.

N-Cam: Blocking buffer: 1% FCS, 1 mg/ml BSA, 1% Triton X 100 in PBS. Primary antibody: Santa Cruz Biotech, SC-1507 used at a concentration of 1:20. Secondary antibody: FITC conjugated rabbit anti-goat IgG (Sigma) used at a dilution of 1:700.

NF200: NF200 was detected by indirect immunofluorescence as described by Robertson (1987). Primary antibody: anti-neurofilament 200 (Sigma Immunochemicals N-4142) used at a dilution of 1:200. Secondary antibody: FITC-conjugated anti-rabbit IgG (Silenus) used at a dilution of 1:60.

Results

Programmed formation of ectodermal, neurectodermal and neural lineages by pluripotent cell differentiation in vitro

EBMs, formed by aggregating ES cells in DMEM + 50% MEDII for 4 days in

suspension culture (Example 1), were maintained in DMEM + 50% MEDII, or DMEM + 50% MEDII + 20 ng/ml FGF4 for a further 3 days in suspension. Under these conditions approaching 100% of EBMs developed into cellular aggregates with a distinctive layered structure of pseudo-stratified epithelial appearance (Figure 31A). These layers were observed in aggregates cultured in 50% MEDII alone but were enhanced in the presence of FGF4. However, these layers were never seen in EPL cell EBs, and were observed only sporadically in ES cell EBs where they comprised a subset of cells within the body.

The identity of cells within these layers was tested by analysis of the expression of transcripts and cell surface markers diagnostic for embryonic cell types. For the analysis of gene expression by in situ hybridisation, aggregates were seeded after 7 days of suspension culture for 16 hours onto gelatin treated tissue culture plastic in DMEM + 50% MEDII +/- FGF4 (Figure 31B). Expression of the pluripotent cell marker *Oct4* could not be detected in the cell layers indicating that these cells were not pluripotent. *Brachyury* expression was not detected by in situ hybridisation, demonstrating that these structures did not contain nascent mesoderm. Further, no cell types of mesodermal morphology could be recognised within the aggregates.

Sox1 is first expressed by the neurectoderm/neural plate in vivo and by all undifferentiated neural cells. It is not expressed in cell types of mesodermal or endodermal origin. *Gbx2* is expressed in the primitive streak, then in the neurectoderm prior to neural tube closure and subsequently at the midbrain/hindbrain boundary. *Sox2* is expressed in the neurectoderm after neural tube closure and persists until terminal differentiation of neural cells. In situ hybridisation demonstrated widespread expression of *Sox1* by cells within the layers, and *Gbx2* by a subset of these cells. This suggested that the cell type produced by these culture conditions was equivalent to neurectoderm, specifically early neural plate around the time of neural tube closure.

The analysis of neural gene expression within these aggregates was refined by analysis of neural specific markers by in situ hybridisation (*Sox1, Sox2* and *Gbx2*; Figure 32A) and immunohistochemistry (nestin and N-CAM; Figure

32B) on days 8, 9 and 10. *Sox1* expression was detected in greater than 90% of aggregates on day 8 of development, however, expression was seen in only a proportion of cells in each aggregate. *Sox1* expression increased on day 9 and 10 of development, with approaching 100% of cells within each aggregate expressing *Sox1*. Similarly, on day 10 the majority of cells were also expressing *Sox2*. *Gbx2* expression was observed in a proportion of cells on day 8 of development and was down-regulated on days 9 and 10. This expression pattern is consistent with identification of the cell layers as neurectodermal in origin.

The identification of these cells as neurectoderm was supported by the expression of neural proteins (Figure 32B, data not shown). Nestin, an intermediate filament protein expressed by undifferentiated neural stem cells, was expressed in the cell layers at days 8, 9 and 10, similar to *Sox1*. N-Cam is a marker expressed by all neural lineages, including both undifferentiated and differentiated cells. N-Cam staining was detected throughout the aggregates on days 8, 9, and 10, with the number of cells expressing N-Cam increasing to nearly 100%. N-Cam was expressed both in the cell layers and in differentiated cells surrounding the layers, indicating that the majority of the differentiated cells were of neural origin.

Northern blot and RNase protection analysis of gene expression supported the conclusion that EBMs were programmed to form neurectoderm in the presence of MEDII. *Oct4* and *Fgf5* expression (Figure 33A) were markedly down regulated in EBM aggregates between day 4 and day 5, indicating the loss of pluripotent cells. However, a low but consistent level of *Oct4* transcript was detected in EBM aggregates after day 5. In situ hybridisation (Figure 31B) demonstrated that the low *Oct4* transcription was not expressed from residual pluripotent cells within the aggregate and may be a feature of the early neurectoderm in these aggregates. This residual expression could not be detected in ES cell EBs which form low levels of neurectoderm. Expression of *brachyury* was observed from day 4 in ES cell EBs, consistent with the loss of Oct4 expression, but could not be detected in EBMs (Figure 8). Finally, *Gbx2* and *Sox1* expression (Figure 33B) were detected by RNase protection in aggregates

formed from EBMs on days 6, 7 and 8. While expression of *Gbx2* decreased on day 8, *Sox1* expression continued to increase to day 10.

These results demonstrate that pluripotent cells can be programmed specifically to an ectodermal and neurectodermal fate by factors within MEDII. The ectodermal cells are formed in the absence of mesodermal cell types, and exhibit a temporal pattern of gene expression equivalent to neurectoderm in vivo, with progression from an early neurectodermal cell type equivalent to neural plate, to a later neurectodermal cell type present after neural tube closure. In the embryo these cells are precursors for all neural lineages.

Neurectoderm derived by directed pluripotent cell differentiation in vitro can be further differentiated to neural cell types.

Individual aggregates developed from EBMs in the presence of MEDII, or ES cell EBs, were seeded and assessed on days 8, 10 and 12 for the presence of neurons, identified morphologically by the presence of axonal projections (and confirmed by the expression of NF200; data not shown), and beating cardiocytes. Beating cardiocytes (Figure 34A) could be detected in over 50% of ES cell EBs on day 8, and these persisted through days 10 and 12 of development. In contrast, the vast majority of EBM-derived aggregates (99.6%) maintained in MEDII did not contain beating cardiocytes. The lack of beating cardiocytes, a mesodermally derived tissue, in these aggregates, is consistent with the lack of *brachyury* expression at earlier stages of development (Example 1, Figure 7, 8) and indicates that mesodermal lineages are not formed in these aggregates. Neurons were not detected in either population of aggregates on day 8 of development (Figure 34B), but were apparent in over 60% of EBM-derived aggregates on day 10. By day 12, in excess of 90% of these aggregates contained neurons. By comparison, formation of neurons was observed in 10% (day 10) and 25% (day 12) of ES cell EBs. These data support the earlier conclusion that neurectoderm is formed at high levels and in the absence of mesodermal cells by EBMs cultured in the presence of MEDII, and demonstrate that this neurectoderm can give rise to terminally differentiated neural cell types. Aggregates developed from EBMs in

MEDII are therefore enriched in undifferentiated neural cells.

Neurectoderm formation in vivo proceeds via the formation of definitive ectoderm. Although no markers exist for definitive ectoderm in vivo, gene expression analysis identified a population of cells in EBMs programmed to form neurectoderm which expressed low levels of *Oct4* and failed to express neurectodermal markers. These cells were present transiently between primitive ectoderm (high *Oct4* expression) and neurectoderm (high *Sox1*, *Gbx2*) and suggest that neurectoderm formation proceeds via an intermediate population which may represent definitive ectoderm.

The formation of neurectoderm described here does not rely on the addition of chemical inducers, such as retinoic acid, or genetic manipulation to promote neural formation. Instead, it relies on biologically derived factors found within the conditioned medium MEDII. Neural progenitors formed in this manner are thought to be differentiated from pluripotent cells in a manner analogous to the formation of neural cells during embryogenesis and are therefore ideal for the production of differentiated neural cells useful for commercial, medical and agricultural applications. Further, in contrast to the formation of limited neural lineages by chemical inducers such as retinoic acid, the identity of neural cell types produced using these methodologies is not likely to be restricted.

Neurectoderm programming of pluripotent cell differentiation requires the large molecular weight components, but not the small molecular weight components, of MEDII.

During embryogenesis neurectoderm forms from anterior primitive ectoderm cells that maintain association with the basement membrane/ extracellular matrix. Identification of a bioactive ECM protein within the large molecular weight component of MEDII prompted an investigation of the role of this activity in directed neurectoderm formation from pluripotent cells. This was tested by the ability of this component to induce formation of neurons within EPL EBs in which neural cell types are not normally formed (Example 6).

EPL cells were formed by culturing ES cells for 2 days in the presence of MEDII and in the absence of LIF. EPL cell EBs were formed in DMEM or DMEM + R (Example 4). On day 4 of development embryoid bodies were individually seeded into gelatin treated 2 ml tissue culture wells. Beating cardiocytes and neurons were assessed on days 7, 8, 9 and 10 of development (Figure 35).

EPL cell EBs formed beating muscle with high efficiency in the absence of the large molecular weight component and none of these EBs contained neurons. Inclusion of the large molecular weight component (50-100 μg/ml) during embryoid body formation resulted in the development of neural cells in about 5% of the EBs at day 9, and in 10-12% at d 10 and 12. Only 10-12% of EPL cell EBs formed in the presence of the large molecular weight component contained beating muscle, and the formation of beating muscle was delayed until day 9. These experiments were carried out in the presence of 1ug/ml neutralising anti-human LIF antibody (R&D Systems). The altered developmental program could therefore not be attributed to low levels of LIF expressed by the Hep G2 cells.

This experiment demonstrates a role for the large molecular weight component of MEDII, and possibly ECM components, in programming of differentiation within pluripotent cell aggregates so as to delay and reduce mesoderm formation, and promote the formation of neurons.

Efficient programmed formation of mesodermal lineages from pluripotent cells

Pluripotent cells can be programmed to adopt a specific mesodermal fate by differentiation through a homogeneous EPL cell intermediate as shown in Example 6. This may reflect the absence of visceral endoderm within EPL cell EBs. Nascent mesoderm formation can be detected in approaching 100% of EPL cell EBs and is probably underestimated by scoring beating cardiocytes which comprise only one of the possible nascent mesoderm cell fates. The previous experiments were carried out using EPL cells formed from ES cells in adherent culture. Here we describe alternative methodologies for efficient mesoderm formation from pluripotent cells in vitro.

Formation of mesoderm by reaggregation of EPL cells formed in suspension

EPL cells formed in suspension in the presence of MEDII (EBMs, Example 1) comprise a stable and homogeneous population of cells with properties equivalent to embryonic primitive ectoderm. Programming the differentiation of these cells to a mesodermal fate is thought to require both removal of the neurectoderm-inducing activity of MEDII and dissociation from ECM components. This would mimic the behaviour of pluripotent cells destined to form mesoderm during primitive streak formation in vivo.

On day 4 EBMs were trypsinised to a single cell suspension and reaggregated in DMEM +/- 10 ng/ml FGF4, or DMEM + 50% MEDII +/- 10 ng/ml FGF4. After a subsequent 4 days of development an obvious morphological difference was apparent between reaggregates cultured in DMEM and those cultured in DMEM + 50% MEDII (Figure 36). RNA from these aggregates on days 2 and 4 was analysed by northern blot for expression of *brachyury* and *Oct4* (Figure 36). *Oct4* expression was down regulated in reaggregates on day 4, indicating the loss of pluripotence as differentiation occurred. *Brachyury* expression could not be detected in reaggregates developed in DMEM + 50% MEDII, consistent with the fact that these are destined to form neurectoderm. *Brachyury* expression, indicating mesoderm formation, was strongly up regulated in reaggregates developed in DMEM at day 2 and 4 in the absence of FGF4. In the presence of FGF4 *brachyury* expression peaked on day 2 and was down regulated by day 4, suggesting that addition of FGF4 resulted in accelerated mesodermal differentiation of the pluripotent cells. The expression of marker genes was confirmed by in situ hybridisation of reaggregated bodies with *Oct4* and *brachyury* specific probes (data not shown). These data indicate that EPL cells formed in suspension can be programmed to differentiate into mesoderm.

Mesodermal precursors derived by directed pluripotent cell differentiation can be programmed to alternative developmental fates

Programmed formation of high levels of mesoderm in EPL cell EBs was demonstrated by formation of nascent mesoderm and beating cardiocytes (Example 6). To establish whether the elevated levels of nascent mesoderm in

EPL cell EBs were developmentally restricted to myogenic lineages or could be programmed to alternative developmental fates, the formation of macrophages in response to exogenous cytokines was assessed.

EPL cell and control ES cell EBs were differentiated in MC media supplemented with mIL-3 and hM-CSF, and scored for the presence of macrophages on days 12, 15 and 18 (Figure 37). On day 12, 32.4% of EPL cell EBs were observed to contain macrophages, compared to 4.8% of ES cell EBs. On days 15 and 18 the proportion of embryoid bodies containing macrophages had increased to 43% of EPL cell EBs and 9-10 % of ES cell EBs. Consistent with the earlier formation of mesoderm in EPL cell EBs, formation of macrophages initiated approximately two days earlier in these embryoid bodies compared to ES cell EBs (data not shown). Enhanced formation of multiple mesodermal lineages in EPL cell EBs suggests that the elevated nascent mesoderm in these bodies contains a multipotent mesodermal progenitor which can be programmed to alternative fates in response to specific environmental cues.

Pluripotent cell differentiation can be specifically programmed to the formation of alternative mesodermal and ectodermal lineages

A comparative analysis of methodologies developed for directed mesodermal (formation of EPL cell EBs) and ectodermal (formation of EBMs) differentiation of pluripotent cells is presented in figure 38. Differentiated aggregates were scored for the presence of neurons and beating muscle as representative cell types for mesodermal and ectodermal lineages respectively.

EPL cell EBs formed beating muscle with high efficiency but were unable to form neurons. EBMs showed the reciprocal pattern, forming neurons with high efficiency but failing to form beating muscle. The formation of mesoderm in particular is probably underestimated in this analysis because only a single mesodermal cell type, beating muscle, was scored. Appropriate use of the factors within MEDII to form and differentiate pluripotent cell populations can therefore be used to direct specific formation of alternative mesoderm and ectoderm germ layers from pluripotent cells in vitro.

Summary

We have described methods for directing the differentiation of pluripotent cells into cell lineages of alternative germ layers, namely ectoderm and mesoderm. Formation of EPL cells in response to the biological activity in MEDII, in adherent or suspension cultures, is an essential step in these lineage specific differentiation protocols. Although the pluripotent cells used in the above examples were obtained by culture of ES cells in MEDII, we envisage that primary pluripotent cells isolated from embryonic ICM, primitive ectoderm or PGCs, or pluripotent cells obtained by dedifferentiation or nuclear transfer, could be used as a source of material for controlled lineage specific differentiation in vitro.

Neurectoderm formation, in the absence of mesoderm formation, occurred when EPL cells were differentiated in the presence of MEDII in suspension culture. This suggested that the conditioned medium contained components required for neural determination. Fractionation of the conditioned medium suggested that this component resided in a fraction containing medium components of greater than 10 kDa, and may be the ECM component identified as active in the formation of EPL cells. Formation of neurectoderm from primitive ectoderm in contact with ECM components is consistent with the determination of this lineage during embryogenesis.

Efficient formation of mesoderm from EPL cells, in the absence of neurectoderm, could be achieved in adherent or suspension culture and required removal of MEDII, potentially to eliminate contact with ECM proteins. This could be achieved by aggregating, or reaggregating, EPL cells in medium without the addition of MEDII, or seeding EPL cells onto gelatin treated plastic-ware in the presence of members of the TGFβ or FGF families. Differentiation of EPL cells as cellular aggregates proceeded in the absence of visceral endoderm and resulted in the formation of mesodermal cell lineages, as assessed by gene expression and terminal differentiation.

Importantly EPL cells, and the inclusion or withdrawal of MEDII or MEDII activity, provide an approach for the production cell populations highly enriched in

progenitor and differentiated cells derived from a single germ layer. This approach does not involve the addition of chemical inducers (such as retinoic acid for neural cell formation) or prior genetic modification of the pluripotent cells. Instead it relies on the factors thought to be involved in neurectoderm and mesoderm formation during normal embryonic development in vivo. Progenitors derived in this manner are ideal for projected applications in human medicine, veterinary applications and agriculture.

EXAMPLE 8:

Gene Therapy Applications

The utility of the present invention in gene therapy is as follows. I depends on using nuclear transfer technology that has already been developed and used in several animal species, including sheep, cattle and mice. A donor human egg is fertilised in vitro, but then the nucleus removed and replaced by a nucleus from a cell from the recipient of the intended gene therapy. The nuclear transferred egg is allowed to develop into an early embryo in vitro, and then cells from it are used to generate, using the procedures and products of this invention, an EPL or ES cell line. This cell line is then genetically modified, using DNA transfer technology known to those skilled in the art, to correct a genetic defect, or to replace or add a functioning copy of a desired gene; (examples; clotting factor genes for hemophelia, the CF gene for cystic fibrosis, the insulin gene for Type 1 diabetes). The modified ES or EPL cell line is then differentiated in vitro to a desired cell, tissue or organ, which is reintroduced into the recipient in a therapeutic transplantation procedure. Since all the genes of the modified cell line (with the exception of the modification or the introduced gene) are identical to the recipient individual's own genes, there is unlikely to be cell or tissue rejection, since this would be in effect, a perfectly matched transplant.

Alternatively, the modified cells from a non-matched human ES or EPL cell line are genetically modified and then encapsulated, by cell encapsulation techniques known to those skilled in the art. They are then introduced to a recipient as an implant to produce in that individual, a gene product (e.g. clotting

factor, insulin, etc.,) missing or defective in the treated individual. It is also possible to genetically modify a donor established ES or EPL cell line to render it non-immunologic to any recipient, thereby generating a "universal ES or EPL cell line". This cell line is further modified to use in the therapeutic procedures described above, without the use of encapsulation technology.

Such "universal" donor cells are used to generate human endothelial cells, that are used in xenotransplantation procedures, for example, to coat xenotransplant organ blood vessels; e.g. pig kidneys or hearts, with human endothelium, to reduce or eliminate delayed graft rejection.

EXAMPLE 9

Transplantation of neural cells derived from pluripotent cells.

Despite the promises offered by neural cell transplantation as a long-term therapy and cure for neurological disorders, significant practical hurdles remain. Allogeneic and xenogeneic transplants are still subject to rejection even though immunological protection is afforded by the blood brain barrier. Furthermore the availability of foetal cells, often used in neural transplantation trials, is scarce. Neural cells, including neural stem cells developed by controlled differentiation *in vitro* of pluripotent cells along defined neural pathways offers an ideal source of cells for neural cell therapy and neural transplantation.

In man transplantation of neural dopamine cells has been trialed to relieve some of the symptoms of Parkinson's disease, and neural cell transplantation may also offer benefits to neurodegenerative disorders. As a first step in providing these cells for human therapy, transplantation with neural cells derived from pluripotent cells can be investigated in a mouse model. Differentiation of ES cell lines expressing β-galactosidase directed to a nuclear location (KSF4 cell line) or to the cytoplasm (ROSA 26 cell line) can be directed along the neural pathway using MEDII as described herein. Neurectoderm or differentiated neural cells can be injected into cerebral vesicles of neonates. Survival of the transplanted cells is

indicated by the maintenance of cells expressing β-galactosidase in the brain. The ability of transplanted cells to integrate can be determined by investigating the connections established by marker cells expressing the cytoplasmic form of β-galactosidase (ie cells derived from the ROSA 26 ES cell line).

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Finally, it is to be understood that various alterations, modifications andlor additions may be made without departing from the spirit of the present invention as outlined herein.

TABLE 1

	ES Cells	ICM	EPL Cells	Primitive Ectoderm
Oct-4	+	+	+	+
Alkaline phosphatase	+	+	+	+
Uvomorulin	+	+	+	+
Fgf5	-	-	+	+
Rex-1	high	+	low	-
Alphafetoprotein(AFP)	-	-	-	-
H19	-	-	-	-
Evx1	-	-	-	-
Brachyury	-	-	-	-

TABLE 2

	Blastocysts injected	Pups born	% liveborn	% chimera born
ES; E14TG2a	163	65	40	58
EPL; 2	65	33	50	0
EPL; 4	77	50	65	0
EPL; 2^R	78	22	28	36
EPL; 4^R	74	31	42	58

TABLE 3

Matrix composition	% EPL cell colonies with no associated differentiation
Gelatin (0.2%)	22 %
Laminin	49 %
Plasma Fibronectin	51 %
Collagen IV	59 %
Laminin/fibronectin/collagen IV mix	62.5%

TABLE 4

Maintenance and proliferation of embryonic primitive ectoderm in response to MEDII

Embryo culture conditions	Matrix	Embryo Survival (%)	Pluripotent cell maintenance (%)
DMEM + mLIF	Collagen IV	23.5	0
DMEM + 50% MEDII	Collagen IV	42	23

TABLE 5

Effect of different treatments/chromatography of semi-purified* HEPG2 conditioned media on its ability to convert ES cells into X cells

Treatment	ES to X cell conversion **
no	+
repeated freeze thawing	+
acid (pH2.0)	+
heat (1hr, 100°C)	+
DTT (50mM)	+
Ion exchange:	
Cation or Anion (various buffers/pH's)	
flow through fraction	+
eluted fraction	-
Reverse phase HPLC	
C18 column (0.1%TFA -> 80%acetonitrile)	
flow through fraction	+
eluted fraction	-
C8 column (0.1%TFA -> 80%acetonitrile)	
flow through fraction	+
eluted fraction	-

*The eluate from the 3kD ultrafiltration of HEPG2 conditioned media was used.
** The ability of the treated/chromatographed eluate (plus desalted retentate) to convert ES to X cells was tested using the assay described in the Materials and Methods.

TABLE 6

Amino acid	Active sample Hydrolysed (pmol)	Active sample Unhydrolysed (pmol)	Control sample (pmol)
Aspartic acid	0	not found	3
Glutamic acid	35.2	8.3	6.2
Serine	2.6	6.1	9.8
Histidine	not found	not found	not found
Glycine	54.4	64.9	not found
Threonine	not found	not found	not found
Alanine	403.3	478.5	11
Arginine	not found	not found	not found
Tyrosine	not found	not found	not found
Valine	18.1	23.9	30.9
Methionine	not found	3.3	18.3
Phenyalanine	0	not found	10.4
Isoleucine	29.5	34.6	58.6
Leucine	25.8	27.4	74.3
Lysine	not found	not found	not found
Proline	478.8	558	25.6

TABLE 7

	Minimal Active concentration (μM)	Range tested (μM)	Activity
L-proline	(40)	20-1000	+
AMINO ACID			
D-Proline		30-3475	-
L-Alanine		390-3900	-
L-Lysine		55-5500	-
PROLINE ANALOG			
N-acetly-L-proline		64-636	-
trans-4-hydroxy-L-proline		270-550	-
N-t-boc-L-proline		10-1000	-*
L-pipecholic acid (PCA)		390-15500	-
Sarcosine		10-1120	-*
3,4 dehydro-L-proline		1-500	-*
pyrrolidine		10-1000	-
PEPTIDE			
Pro-ala	(250)	20-1000	+
Ala-pro	(80)	20-1000	+
Ala-pro-gly	(40)	40-1000	+
Pro-OH-pro	(40-80)	20-1000	+
Pro-gly	(250*)	20-1000	+
Gly-pro	(40)	20-1000	+
Gly-pro-ala	(40)	20-1000	+
Gly-pro-OH-pro	(300)	40-5850	+
Gly-pro-arg-pro	(80)	40-1000	+
Gly-pro-gly-gly	(50)	1-1200	+
Val-ala-pro-gly	(40)	40-1000	+
Substance P (Arg-pro-lys-pro-gln-gln-phe-phe-gly-leu-met-NH_2)		0.005-500	-*
Substance P free acid (Arg-pro-lys-pro-gln-gln-phe-phe-gly-leu-met-OH)	(40)	40-1000	+
Substance P frag. 1-4 (Arg-pro-lys-pro)	(40)	40-1000	+
Arg-gly-asp (RGD)		40-4800	-

TABLE 8

Matrix	Matrix Presentation			
	Solution	Solution + L-proline	Matrix	Matrix + L-proline
Collagen IV	-	-	-	-
Vitronectin	-	-	-	-
Gelatin	-	-	-	-
Laminin	-	-	-	+
Plasma Fibronectin	-	-	+/-	+
Cellular Fibronectin	-	+	+/-	+
Purified bioactivity	-	+	+/-	+
MEDII	+	+	+/-	+

Claims

1. A partially or substantially purified biologically active factor, capable in the presence or absence of additional gp130 agonist of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said factor including a low molecular weight component selected from the group consisting of proline and functionally active analogues thereof, peptides including proline and functionally active fragments and analogues thereof; and molecules which compete therewith for biological activity; and a large molecular weight component selected from the group consisting of extracellular matrix proteins and functionally active fragments or analogues thereof; and molecules which compete therewith for biological activity.

2. A biologically active factor according to Claim 1 wherein the low molecular weight component has a molecular weight of less than approximately 5 kD and the large molecular weight component has a molecular weight of greater than approximately 10 kD.

3. A biologically active factor according to Claim 1 wherein the low molecular weight component is selected from the group consisting of:

proline
Pro-ala
Ala-pro
Ala-pro-gly
Pro-OH-pro
Pro-gly
Gly-pro
Gly-pro-ala
Gly-pro-OH-pro
Gly-pro-arg-pro
Gly-pro-gly-gly
Val-ala-pro-gly

Substance P frag. 1-4 (Arg-pro-lys-pro)

Substance P free acid

(arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-gly-Leu-metOH)

Protease digested (including collagenase digested) collagen fragments; and functionally active fragments and analogues thereof; and molecules which complete therewith for biological activity.

4. A biologically active factor according to Claim 1 wherein the large molecular weight component is a fibronectin or a functionally active fragment or analogue thereof or laminin or functionally active fragment or analogue thereof.

5. A biologically active factor according to Claim 4 wherein the large molecular weight component is a cellular fibronectin or functionally active fragment or analogue thereof.

6. A partially or substantially purified cellular fibronectin or functionally active fragment or analogue thereof capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells

7. A composition capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said composition including a biologically active factor according to Claim 1 or the low or large molecular weight component thereof, and an adjuvant, diluent or carrier therefor.

8. A method for preparing a conditioned medium capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said method including

providing

cells selected from the group consisting of hepatic or hepatoma cells, hepatic or hepatoma cell lines, extraembryonic endodermal cells and cell lines, and

a cell culture medium;

culturing the cells in the cell culture medium for a time sufficient to produce the conditioned medium; and

separating the cells from the culture medium to provide the conditioned medium.

9. A method according to Claim 8 wherein the cells or cell lines are selected from the group consisting of a human or mouse hepatocellular carcinoma cell lines, primary embryonic mouse liver cells, primary adult mouse liver cells, primary chicken liver cells, and extraembryonic endodermal cells or cell lines.

10. A method according to Claim 9 wherein the cells or cell lines are selected from the group consisting of the human hepatocellular carcinoma cell line Hep G2 (ATCC HB-8065), the mouse hepatocellular carcinoma cell line Hepa 1c1c-7 (ATCC CRL-2026), the visceral endodermal cell line END-2 and the parietal endodermal cell line PYS-2.

11. A conditioned medium capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said conditioned medium including a biologically active factor according to Claim 1 or the low and/or large molecular weight component thereof.

12. A method for preparing an extracellular matrix capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said method including

providing

cells selected from the group consisting of hepatic or hepatoma cells, hepatic or hepatoma cell lines, extraembryonic endodermal cells and cell lines

a support substrate, and

a cell culture medium;

culturing the cells in the cell culture medium in the presence of the support substrate for a time sufficient to produce the extracellular matrix; and

separating the cells and culture medium from the support substrate to provide the extracellular matrix.

13. A method according to Claim 12 wherein the cells or cell line are selected

from the group consisting of a human or mouse hepatocellular carcinoma cell lines, primary embryonic mouse liver cells, primary adult mouse liver cells, primary chicken liver cells, and human and mouse extraembryonic endodermal cells and cell lines.

14. A method according to Claim 13 wherein the cells or cell lines are selected from the group consisting of the human hepatocellular carcinoma cell line Hep G2 (ATCC HB-8065), the mouse hepatocellular carcinoma cell line Hepa 1c1c-7 (ATCC CRL-2026).

15. An extracellular matrix capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said extracellular matrix including a biologically active factor according to Claim 1 or the large molecular weight component thereof.

16. A method for partially or substantially purifying a large molecular weight component of a biologically active factor capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said method including

providing

a source of said large molecular weight component,

a heparin affinity chromatography support,

an anion exchange chromatography support, and

a gel filtration chromatography support;

contacting the source of said large molecular weight component with the heparin affinity chromatography support to produce a first fraction;

contacting said first fraction with the anion exchange chromatography support to produce a second fraction; and

contacting said second fraction with the gel filtration chromatography support to produce the partially or substantially purified large molecular weight component.

17. A method according to Claim 16 wherein the source of said large molecular weight component is a partially purified biologically active factor according to Claim 1, a conditioned medium according to Claim 11 or an extracellular matrix

according to Claim 15.

18. A method according to Claim 17 wherein the heparin affinity chromatography support is a heparin sepharose CL-6B column and the gel filtration chromatography support is a superose 6 gel filtration column.

19. A method for partially or substantially purifying a large molecular weight component of a biologically active factor capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said method including

providing

a source of said large molecular weight component,

an ultrafiltration membrane

an anion exchange chromatography support,

a hydrophobic interaction chromatography support,

a heparin affinity chromatography support, and

a gel filtration chromatography support;

contacting the source of the large molecular weight component with the ultrafiltration membrane to obtain a fraction with components greater than approximately 10 kD,

contacting the fraction with components greater than approximately 10 kD with the anion exchange chromatography support to produce a first fraction;

contacting said first fraction with the hydrophobic interaction chromatography support to produce a second fraction;

contacting said second fraction with the heparin affinity chromatography support to produce a third fraction; and

contacting said third fraction with the gel filtration chromatography support to produce the partially or substantially purified large molecular weight component.

20. A method according to Claim 19 wherein the source of said large molecular weight component is a partially purified biologically active factor according to Claim 1, a conditioned medium according to Claim 11 or an extracellular matrix according to Claim 15.

21. A method according to Claim 20 wherein the ultrafiltration membrane is an

Amicon DiaFlo YM10 membrane, the anion exchange chromatography support is a Separose Q anion exchange column, the hydrophobic interaction chromatography support is a phenyl sepharose hydrophobic interaction column, the heparin affinity chromatography support is a heparin sepharose CL-6B column and the gel filtration chromatography support is a Superose 6 gel filtration column.

22 A method for partially or substantially purifying a large molecular weight component of a biologically active factor capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said method including

providing

a source of said large molecular weight component, and

a gelatin affinity chromatography support,

a dialysis system;

contacting the source of said large molecular weight component with the gelatin affinity chromatography support to produce a first fraction; and

subjecting said first fraction to dialysis to produce the partially or substantially purified large molecular weight component.

23. A method according to Claim 22 wherein the source of said large molecular weight component is a partially purified biologically active factor according to Claim 1, a conditioned medium according to Claim 11 or an extracellular matrix according to Claim 15.

24. A method according to Claim 23 wherein the gelatin affinity chromatography support is a gelatin sepharose affinity chromatography support.

25 A method for partially or substantially purifying a low molecular weight component of a biologically active factor capable of influencing differentiation, proliferation and/or maintenance of pluripotent cells, said method including

providing

a source of said low molecular weight component,

an ultrafiltration membrane,

a first gel filtration chromatography support,

a normal phase chromatography support, and

a second gel filtration chromatography support;

contacting the source of said low molecular weight component with the ultrafiltration membrane to obtain a fraction having components less than about 3 kD;

contacting the fraction with components less than approximately 3 kD with the first gel filtration chromatography support to produce a first fraction;

contacting said first fraction with the normal phase chromatography support to produce a second fraction; and

contacting said second fraction with the second gel filtration chromatography support to produce the partially or substantially purified low molecular weight component.

26 A method according to Claim 25 wherein the source of said low molecular weight component is a partially purified biologically active factor according to Claim 1, or a conditioned medium according to Claim 11.

27 A method according to Claim 25 wherein the ultrafiltration membrane is an Amicon DiaFlo YM3 membrane, the first gel filtration chromatography support is a sepharose gel filtration chromatography support and the second gel filtration chromatography support is a Superdex peptide gel filtration chromatography support.

28. A method of producing and/or maintaining early primitive ectoderm-like (EPL) cells, said method including

providing

pluripotent cells, and

a biologically active factor according to Claim 1 or the large or low molecular weight component thereof; or

a conditioned medium according to Claim 11, or

an extracellular matrix according to Claim 15 and optionally the low molecular weight component; and

contacting the pluripotent cells with the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix, in the presence or absence of additional gp130 agonist, to

produce or maintain the EPL cells.

29. A method according to Claim 28 wherein the pluripotent cells are selected from the group consisting of embryonic stem (ES) cells, *in vivo* or *in vitro* derived ICM/epiblast, in vivo or in vitro derived primitive ectoderm, primordial germ cells, EG Cells, teratocarcinoma cells, EC cells, and pluripotent cells derived by dedifferentiation and/or by nuclear transfer.

30. A method according to Claim 29 28 wherein the low molecular weight component is selected from the group consisting of:

proline
Pro-ala
Ala-pro
Ala-pro-gly
Pro-OH-pro
Pro-gly
Gly-pro
Gly-pro-ala
Gly-pro-OH-pro
Gly-pro-arg-pro
Gly-pro-gly-gly
Val-ala-pro-gly
Substance P frag. 1-4 (Arg-pro-lys-pro)
Substance P free acid
(arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-gly-Leu-metOH)
Protease digested (including collagenase digested) collagen fragments;

and functionally active fragments and analogues thereof; and molecules which compete therewith for biological activity.

31. A method according to Claim 28 wherein the large molecular weight component is a fibronectin or a functionally active fragment or analogue thereof or laminin or functionally active fragment or analogue thereof.

32. A method according to Claim 31 wherein the large molecular weight

component is a cellular fibronectin or a functionally active fragment or analogue thereof.

33. A method according to Claim 28 wherein the pluripotent cells are contacted with the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular, in the presence of additional gp130 agonist.

34. A method according to Claim 28 further including identifying the EPL cells, preferably by *Oct4* and *Fgf5* expression.

35. A method according to Claim 28 wherein the EPL cells are mammalian or avian.

36. A cultured EPL cell.

37. A cell according to Claim 36 which is mammalian or avian.

38. A method of producing partially differentiated and/or terminally differentiated cells, said method including

providing

pluripotent cells, and

a biologically active factor according to Claim 1, or the large or low molecular weight component thereof, or

a conditioned medium according to Claim 11, or

an extracellular matrix according to Claim 15 and optionally the low molecular weight component; and

contacting the pluripotent cells with the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix in the presence or absence of additional gp130 agonist, to produce EPL cells; and

culturing the EPL cells in the presence or absence of the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix, and in the presence or absence of one or

more differentiation agents; to produce the partially differentiated and/or terminally differentiated cells.

39. A method according to Claim 38 wherein the pluripotent cells are selected from the group consisting of embryonic stem (ES) cells, *in vivo* or *in vitro* derived ICM/epiblast, *in vivo* or *in vitro* derived primitive ectoderm, primordial germ cells, EG cells, teratocarcinoma cells, EC cells and pluripotent cells derived by dedifferentiation and/or by nuclear transfer.

40. A method according to Claim 38 wherein the low molecular weight component is selected from the group consisting of:

proline
Pro-ala
Ala-pro
Ala-pro-gly
Pro-OH-pro
Pro-gly
Gly-pro
Gly-pro-ala
Gly-pro-OH-pro
Gly-pro-arg-pro
Gly-pro-gly-gly
Val-ala-pro-gly
Substance P frag. 1-4 (Arg-pro-lys-pro)
Substance P free acid
(arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-gly-Leu-metOH)

Protease digested (including collagenase digested) collagen fragments; and functionally active fragments and analogues thereof; and molecules which compete therewith for biological activity.

41. A method according to Claim 38 wherein the large molecular weight component is a fibronectin or a functionally active fragment or analogue thereof or laminin or functionally active fragment or analogue thereof.

42. A method according to Claim 41 wherein the large molecular weight component is a cellular fibronectin or a functionally active fragment or analogue thereof.

43. A method according to Claim 38 further including identifying the partially differentiated and/or terminally differentiated cells by expression of cell surface markers, morphology and/or differentiation potential.

44. A method according to Claim 38 wherein the partially differentiated and/or terminally differentiated cells are mammalian or avian.

45. A method according to Claim 38 wherein the EPL cells are cultured in suspension in the presence of the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix, preferably in the presence of a growth factor from the FGF family; and the partially differentiated and/or terminally differentiated cells include predominantly ectoderm germ layer cells, predominantly partially differentiated ectoderm, predominantly neurectoderm or predominantly neural stem cells and/or predominantly terminally differentiated dermal or neuronal cells.

46. A method according to Claim 38 wherein the EPL cells are cultured in suspension in the absence of the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix, and the partially differentiated and/or terminally differentiated cells include predominantly mesodermal germ layer cells, predominantly partially differentiated and/or predominantly terminally differentiated nascent mesodermal cells such as blood cells and muscle cells.

47. A method according to Claim 38 wherein EPL cells formed in suspension are disaggregated and reaggregated in the absence of the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix, preferably in the presence of growth factors from the FGF family, and the cells are and the partially differentiated and/or terminally differentiated cells include predominantly mesodermal germ layer cells,

predominantly partially differentiated and/or predominantly terminally differentiated nascent mesodermal cells such as blood cells and muscle cells.

48. A method according to Claim 38 wherein the EPL cells are cultured in adherent culture in the absence of the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix, in the presence of growth factors from the FGF and TGFß families, and the cells are differentiated.

49. A method according to Claim 46 or Claim 47 to differentiate pluripotent cells in the absence of visceral endoderm.

50. A method of producing predominantly ectodermal cells, predominantly neurectodermal cells, and predominantly partially and/or terminally differentiated neurectodermal cells, by pluripotent cell differentiation according to Claim 45.

51 A method of producing predominantly very early or nascent mesodermal cells, and predominantly partially and/or terminally differentiated mesodermal cells, by pluripotent cell differentiation according to Claim 46 or Claim 47.

52 A neurectoderm cell, a partially differentiated neurectodermal or neural stem cell or terminally differentiated neuronal cell produced by the method of Claim 38 or 50.

53 A mesodermal germ layer cell, and a partially differentiated mesodermal cell or terminally differentiated mesodermal cell such as a muscle cell or blood cell produced by the method of Claim 38 or Claim 46 or Claim 47 or claim 51.

54. An ectodermal germ layer cell, a partially differentiated ectodermal cell, or terminally differentiated ectodermal cell such as a dermal cell, produced by the method of Claim 38 or claim 50.

55. A method of producing embryonic stem (ES) cells, said method including providing

pluripotent cells,

a biologically active factor according to Claim 1, or the large or low molecular weight component thereof, or

a conditioned medium according to Claim 11, or

an extracellular matrix according to Claim 15 and optionally the low molecular weight component; and

a gp130 agonist;

contacting the pluripotent cells with the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix, in the presence or absence of additional ~~and the~~ gp130 agonist to produce EPL cells; and

contacting the EPL cells with the gp130 agonist in the absence of the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix, to enable the EPL cells to revert to ES cells.

56. A method according to Claim 55 wherein the pluripotent cells are selected from the group consisting of embryonic stem (ES) cells, *in vivo* or *in vitro* derived ICM/epiblast, in vivo or in vitro derived primitive ectoderm, primordial germ cells, EG cells, teratocarcinoma cells, EC cells and pluripotent cells derived by dedifferentiation and/or by nuclear transfer.

57 A method according to Claim 55 wherein the low molecular weight component is selected from the group consisting of:

proline
Pro-ala
Ala-pro
Ala-pro-gly
Pro-OH-pro
Pro-gly
Gly-pro
Gly-pro-ala
Gly-pro-OH-pro

Gly-pro-arg-pro
Gly-pro-gly-gly
Val-ala-pro-gly
Substance P frag. 1-4 (Arg-pro-lys-pro)
Substance P free acid
(arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-gly-Leu-metOH)

Protease digested (including collagenase digested) collagen fragments; and functionally active fragments and analogues thereof; and molecules which compete therewith for biological activity.

58. A method according to Claim 55 wherein the large molecular weight component is a fibronectin or a functionally active fragment or analogue thereof or laminin or functionally active fragment or analogue thereof.

59. A method according to Claim 58 wherein the large molecular weight component is a cellular fibronectin or a functionally active fragment or analogue thereof.

60. A method according to Claim 55 wherein the ES cells are mammalian or avian.

61. A method according to Claim 55 further including differentiating the ES cells to produce partially or terminally differentiated cells.

62. An ES cell produced by the method of Claim 55

63. A cell according to Claim 62 which is mammalian or avian.

64. A method of producing genetically modified ES cells, said method including providing

pluripotent cells,

a biologically active factor according to Claim 1, or the large or low molecular weight component thereof, or

a conditioned medium according to Claim 11, or

an extracellular matrix according to Claim 15 and optionally the low molecular weight component; and

a gp130 agonist;

contacting the pluripotent cells with the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix, in the presence or absence of additional gp130 agonist to produce EPL cells;

modifying one or more genes in the EPL cells; and

contacting the genetically modified EPL cells with the gp130 agonist in the absence of the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix, to enable the genetically modified EPL cells to revert to genetically modified ES cells.

65. A method of producing genetically modified EPL cells, said method including

providing

pluripotent cells,

a biologically active factor according to Claim 1, or the large or low molecular weight component thereof, or

a conditioned medium according to Claim 11, or

an extracellular matrix according to Claim 15 and optionally the low molecular weight component; and

a gp130 agonist;

modifying one or more genes in the pluripotent cells; and

contacting the genetically modified pluripotent cells with the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix; in the presence or absence of additional gp130 agonist to produce the genetically modified EPL cells.

66. A method according to Claim 65 wherein the pluripotent cells are selected from the group consisting of embryonic stem (ES) cells, ES cells derived according to claim 50, in vivo or in vitro derived ICM/epiblast, in vivo or in vitro

derived primitive ectoderm, primordial germ cells, EG cells, teratocarcinoma cells, EC cells and pluripotent cells derived by dedifferentiation or by nuclear transfer.

67. A method of producing genetically modified EPL cells which method includes producing an EPL cell according to Claim 28 and genetically modifying the EPL cell.

68. A method of producing genetically modified partially or terminally differentiated cells which method includes

providing

pluripotent cells,

a biologically active factor according to Claim 1, or the large or low molecular weight component thereof, or

a conditioned medium according to Claim 11, or

an extracellular matrix according to Claim 15 and optionally the low molecular weight component; and

a gp130 agonist;

contacting the pluripotent cells with the biologically active factor or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix, in the presence or absence of additional gp130 agonist to produce EPL cells;

modifying one or more genes in the EPL cells; and

differentiating the EPL cells to produce the genetically modified partially or terminally differentiated cells.

69. A method of producing genetically modified partially or terminally differentiated cells which method includes preparing partially or terminally differentiated cells according to Claim 38 and genetically modifying the cells.

70. A genetically modified ES cell produced by the method of Claim 64.

71. A genetically modified EPL cell produced by the method of Claim 65 or 66.

72. A genetically modified partially or terminally differentiated cell produced by

the method of Claim 67 or Claim 68.

73 A method of producing a chimeric animal said method including

providing

an ES cell according to Claim 62 or a genetically modified ES cell according to Claim 69, and

a pregastrulation embryo;

introducing the ES cell or genetically modified ES cell into the pregastrulation embryo; and

monitoring chimera forming ability.

74. A chimeric or transgenic animal produced by the method of Claim 73.

75. A method of using unmodified EPL cells according to claims 36 and 37, or their differentiated progeny according to claim 72, for use in human cell therapy and transgenic animal production.

76. A method of using genetically modified EPL cells according to 71 or their differentiated progeny according to claim 72, for use in human gene therapy and transgenic animal production.

77. A method of preparing a nuclear transfer cell, said method including

providing

an EPL cell or partially or fully differentiated cell derived from an EPL cell, and

an enucleated recipient cell;

transferring the EPL cell or partially or fully differentiated cell derived from an EPL cell or nucleus derived from these cells to the enucleated recipient;

fusing and activating the combined cell to form a nuclear transfer cell.

78. A method according to Claim 77 wherein the enucleated recipient cell is an oocyte, single cell embryo or other pluripotent cell.

79. A method for deriving a nuclear transfer cell, said method including

providing

a cell,

a recipient enucleated EPL cell or enucleated cell derived from an EPL cell;

transferring the cell to the enucleated EPL cell or enucleated cell derived from an EPL cell;

fusing and activating the combined cell to form a nuclear transfer cell.

80. Nuclear transfer cells as derived according to Claims 77 and 79.

81. Mammals and birds derived from the nuclear transfer cells according to Claim 80.

82. Pluripotent cells derived from any nuclear transfer cell according to Claim 81.

FIGURE 1

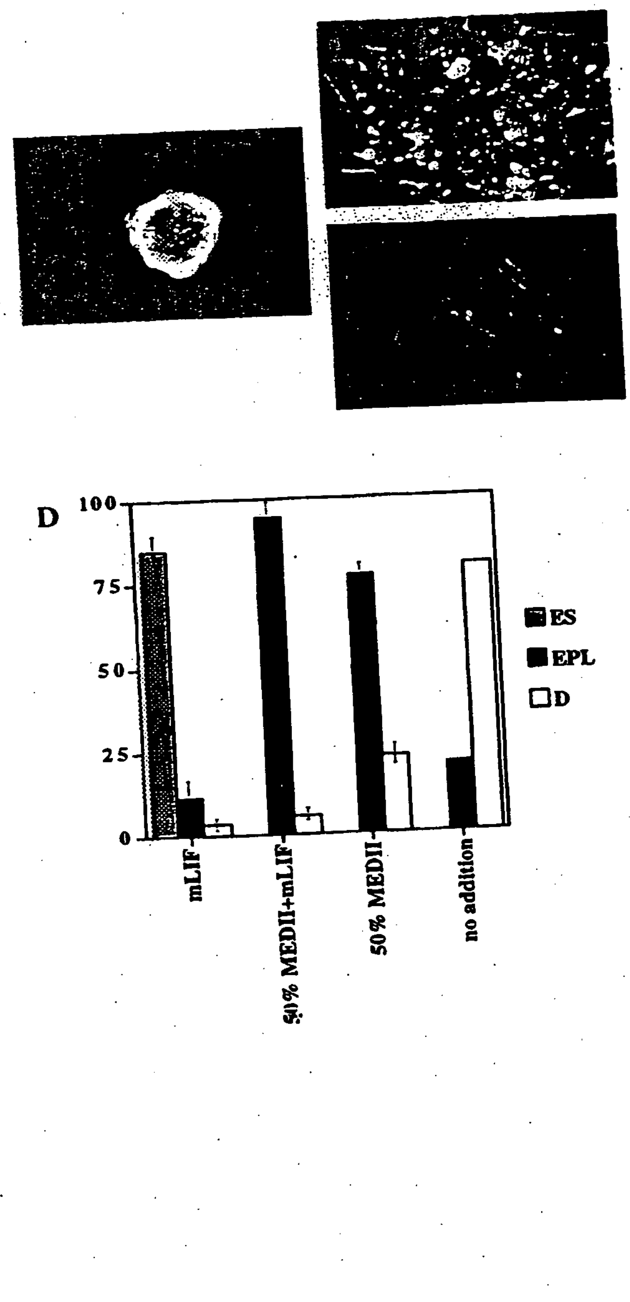


FIGURE 2

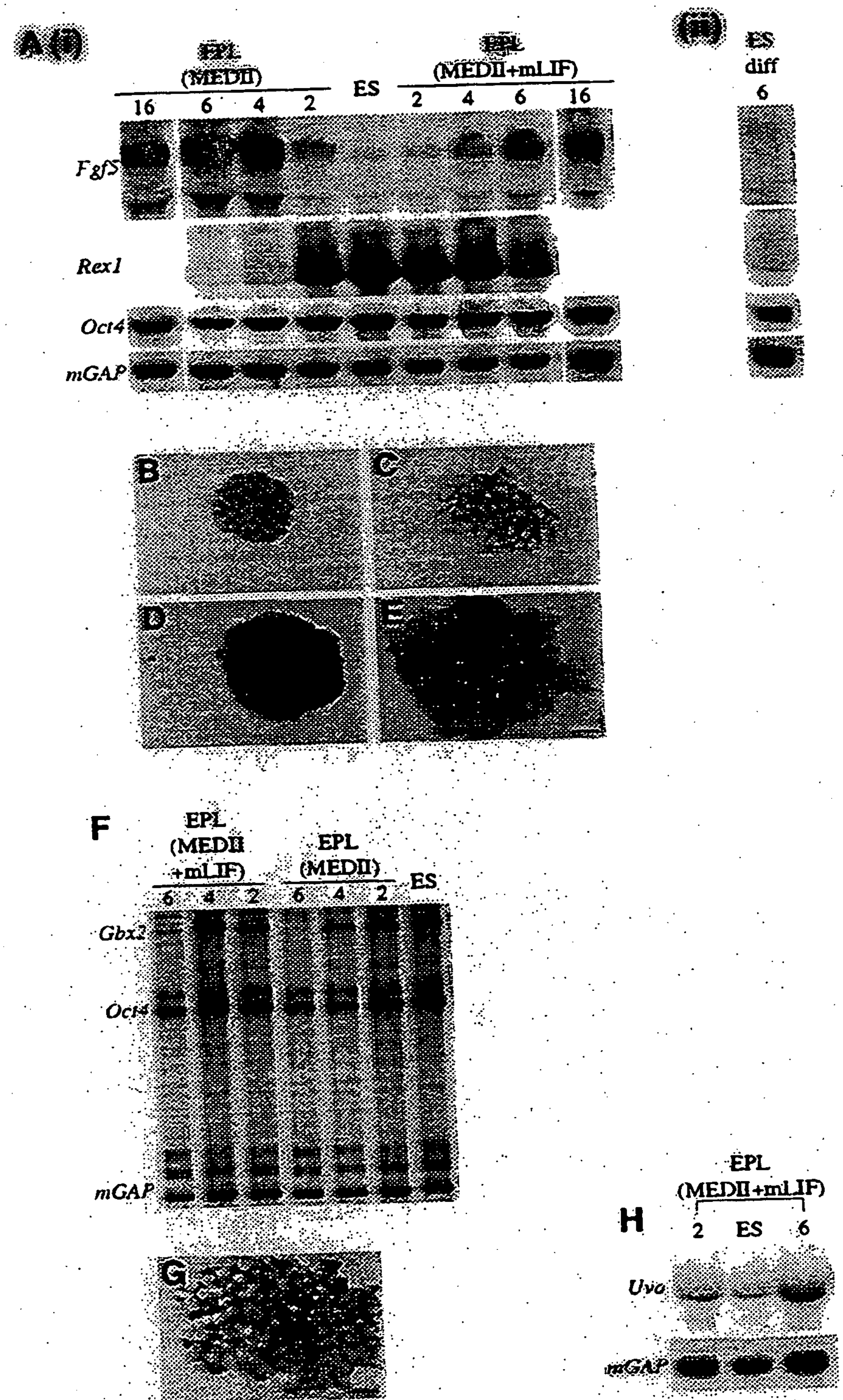

A (i)
EPL
(MEDII)
16
6
4
2
ES
EPL
(MEDII+mLIF)
2
4
6
16
Fgf5
Rex1
Oct4
mGAP
(ii)
ES
diff
6
B
C
D
E
F
EPL
(MEDII
+mLIF)
EPL
(MEDII)
ES
6
4
2
6
4
2
Gbx2
Oct4
mGAP
G
H
EPL
(MEDII+mLIF)
2
ES
6
Uvo
mGAP

FIGURE 3A

L17 DDPCR product Sequence :

```
        10         20         30         40         50         60
TCTATACCTG ACTGCATGGA ACGGGTCACA TTCAAAACAT GGATATGCTA CAAACAAAAT
AGATATGGAC TGACGTACCT TGCCCAGTGT AAGTTTTGTA CCTATACGAT GTTTGTTTTA

        70         80         90        100        110        120
TACCCTTGGG TCATGTGTGC AGCATATGCA GAAACACAAG TGGATCTTGT GTGTGGATGT
ATGGGAACCC AGTACACACG TCGTATACGT CTTTGTGTTC ACCTAGAACA CACACCTACA

       130        140        150        160        170        180
AAACCCCATC CCCAAGATAA CTGTATTACA TTTGTATGTA TTGGTAGTTC AAGATCTCAA
TTTGGGGTAG GGGTTCTATT GACATAATGT AAACATACAT AACCATCAAG TTCTAGAGTT

       190        200        210        220        230        240
CCCGCCCAGG TAGAGGCTTG TTTATCTAGG ATATTCAACC TTCCGTGGAC TGAGCCAGGT
GGGCGGGTCC ATCTCCGAAC AAATAGATCC TATAAGTTGG AAGGCACCTG ACTCGGTCCA

       250        260        270        280        290        300
CCTACGAAGG CCACATCTGT GAAGTACGTG GACAAGCTTC TTCCCACGTG GCTGCCCTGA
GGATGCTTCC GGTGTAGACA CTTCATGCAC CTGTTCGAAG AAGGGTGCAC CGACGGGACT

       310        320        330        340        350        360
GGCTTTGTCA CTGGGCTGAC GGAACTACGG GAGAGAGCTT TGTGGGGCTA GTATTAGAGA
CCGAAACAGT GACCCGACTG CCTTGATGCC CTCTCTCGAA ACACCCCGAT CATAATCTCT

       370        380        390        400        410        420
CAAGCAAAGG GGGACCATTT CAAGCACTGA CCTATCTCTT CCTGCTGGGT AAAGTCTTGC
GTTCGTTTCC CCCTGGTAAA GTTCGTGACT GGATAGAGAA GGACGACCCA TTTCAGAACG

       430        440        450        460        470        480
TTACTTACCA TGTTCTTCCA TGCTTTGATG TGACTCTTTC TTGAGGTGTT CTCACCTACC
AATGAATGGT ACAAGAAGGT ACGAAACTAC ACTGAGAAAG AACTCCACAA GAGTGGATGG

       490        500        510        520        530        540
TTCTTCCTCT AGGTGGGGTG ATTCTTTTCT AAAGGTGGTA GGGTTTTTTA GGTGGTAGTC
AAGAAGGAGA TCCACCCCAC TAAGAAAAGA TTTCCACCAT CCCAAAAAAT CCACCATCAG

       550        560        570        580        590        600
CAGACTGGTC AAATATGTGT GGCAGGTCAC GGGAAAGGGA GTCATGGAAA CCCACGTTGT
GTCTGACCAG TTTATACACA CCGTCCAGTG CCCTTTCCCT CAGTACCTTT GGGTGCAACA

       610        620        630        640        650        660
CATAATGCCA TGTTGTTTTT GTACCTGAAA TAAAGCATAT TTTGCACTTG TAAATGAAAA
GTATTACGGT ACAACAAAAA CATGGACTTT ATTTCGTATA AAACGTGAAC ATTTACTTTT

       670        680        690        700        710        720
ATCTGTATGT GGGCTCTGTG CTGGGTCAGA ATGCAAATAA AACCATTTGT ACTAAAAAAA
TAGACATACA CCCGAGACAC GACCCAGTCT TACGTTTATT TTGGTAAACA TGATTTTTTT

       730        740        750        760        770        780
AAAAAAAAAA AAAAAA.... .......... .......... .......... ..........
TTTTTTTTTT TTTTTT.... .......... .......... .......... ..........
```

FIGURE 3B

K7 DD-PCR Product Sequence

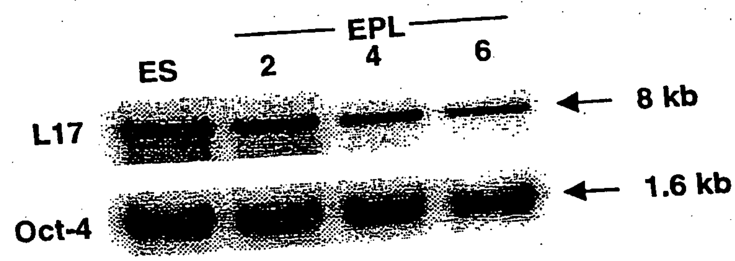
```
        10         20         30         40         50         60
CAGGCCCTTC GACTCAAGTA CCTCATTGGT GCTGCACCTG TACTACGGCC TACCTATCTC
GTCCGGGAAG CTGAGTTCAT GGAGTAACCA CGACGTGGAC ATGATGCCGG ATGGATAGAG

        70         80         90        100        110        120
TCTGCAGACA CCCTGAAGCT GTCTTGTCTA TGGTAGAAGC TGTGTGACTA CCTCCAAACT
AGACGTCTGT GGGACTTCGA CAGAACAGAT ACCATCTTCG ACACACTGAT GGAGGTTTGA

       130        140        150        160        170        180
TGGTTTTGAT TATTTAGAAT TTTTAAAGTG ATTTTCCCTG GTGTTTTGTA TGAAATATTT
ACCAAAACTA ATAAATCTTA AAAATTTCAC TAAAAGGGAC CACAAAACAT ACTTTATAAA

       190        200        210        220        230        240
TCTTTAATGT GACCTTAATA AAGATATTCC AGGCCCTTCT AAAAAAAAAA AA........
AGAAATTACA CTGGAATTAT TTCTATAAGG TCCGGGAAGA TTTTTTTTTT TT........
```

FIGURE 3C

Psc1 DD-PCR Product Sequence

```
                 3'-hp
B04/400          GAATTCCGACGGTTTTGGAACCA GAG AGC CGA AAG AGA GCT ATT AGT GGT TTG GAG GGG CCA CTT   65
ORF                                       E   S   R   K   R   A   I   S   G   L   E   G   P   L    14
CLEB0336.3 10                             A   A   Q   G   V   V   T   Q   P   G   S   F   R   G   338

ACA AAG AAG CCT TGG CTG GGA AAA CAA GGG AAC AAC AAT CAA AGT AAA CCA GGC TTC CTG  125
 T   K   K   P   W   L   G   K   Q  [G]  N   N   N   Q   S   K   P  [G   F]  L    34
 A   P   R   G   R   G   N   I   R  [G]  R   G   G   P   A   R   G  [G   F]  T   358

AGG AAG AAT CAT TAT ACA AAC ACC AAA CTA GAG GTG AAA AAA ATC CCC CAG GAA TTG AAC  185
 R   K   N   H   Y   T  [N]  T   K  [L]  E  [V]  K  [K   I   P]  Q  [E]  L  [N]   54
 G   A   I   N   R   D  [N]  C   T  [L]  Q  [V]  A  [K   I   P]  P  [E]  M  [N]  378

AAC ATT ACC AAG CTC AAC GAA CAC TTC AGC AAA TTT GGA ACC ATA GTT AAT ATC CAG GTT  245
 N  [I]  T  [K   L   N   E   H   F]  S   K  [F   G   T]  I   V  [N   I   Q   V]   74
 T  [I]  A  [K   L   N   E   H   F]  A   T  [F   G   T]  V   D  [N   I   Q   V]  398

GCT TTT AAG GGT GAT CCA GAA GCA GCA CTC ATC CAA TAC CTT ACC AAT GAA GAG GCC AGG  305
 A   F   K  [G]  D   P   E   A  [A   L]  I   Q   Y   L   T   N   E   E  [A]  R    94
 R   Y   N  [G]  E   I   D   S  [A   L]  V   T   Y   A   S   K   F   D  [A]  G   418

AAA GCC ATT TCT AGC ACA GAA GCA GTT TTG AAC AAT CGG TTC ATT CGA GTC CTG TGG CAT  365
[K   A]  I   S  [S]  T   E   A  [V   L   N   N   R   F   I]  R  [V]  L  [W   H]  114
[K   A]  Y   K  [S]  P   T   P  [V   L   N   N   R   F   I]  K  [V]  F  [W   H]  438

                                                                                  458
AGA GAG AAC AAT GAG CAA CCA GCACTCCAGTCC                                         121
 R   E   N   N  [E]  Q   P   OPB-04                                               445
 N   P   G   G  [E]  G   S
```

FIGURE 4A


EPL
ES
2
4
6
L17
Oct-4
8 kb
1.6 kb

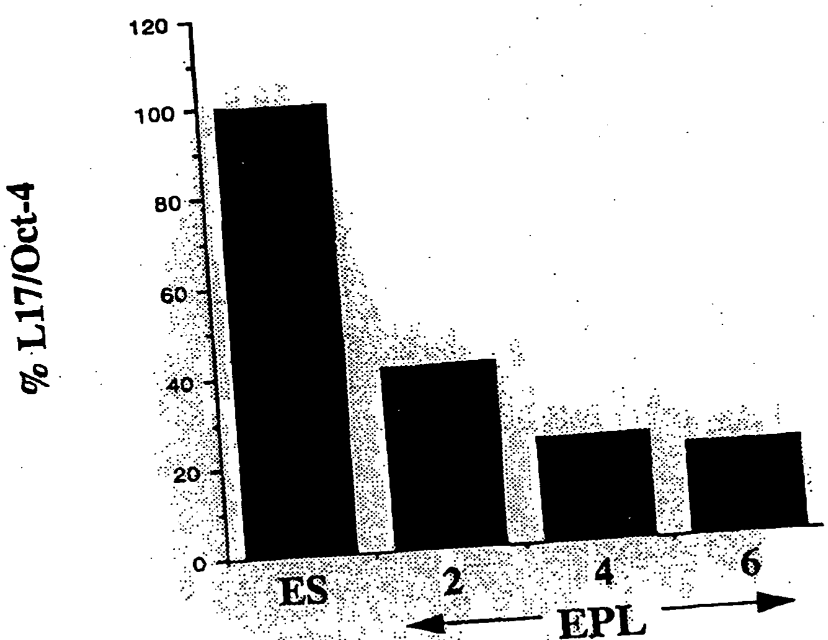
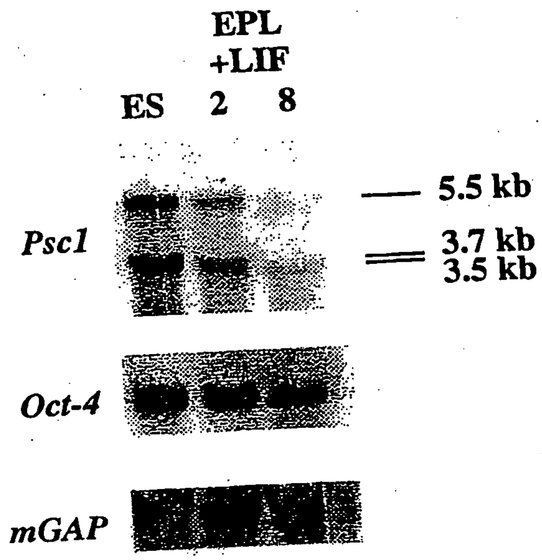

% L17/Oct-4
120
100
80
60
40
20
0
ES
2
4
6
EPL

FIGURE 4B


EPL
+LIF
ES 2 8
Pscl
5.5 kb
3.7 kb
3.5 kb
Oct-4
mGAP

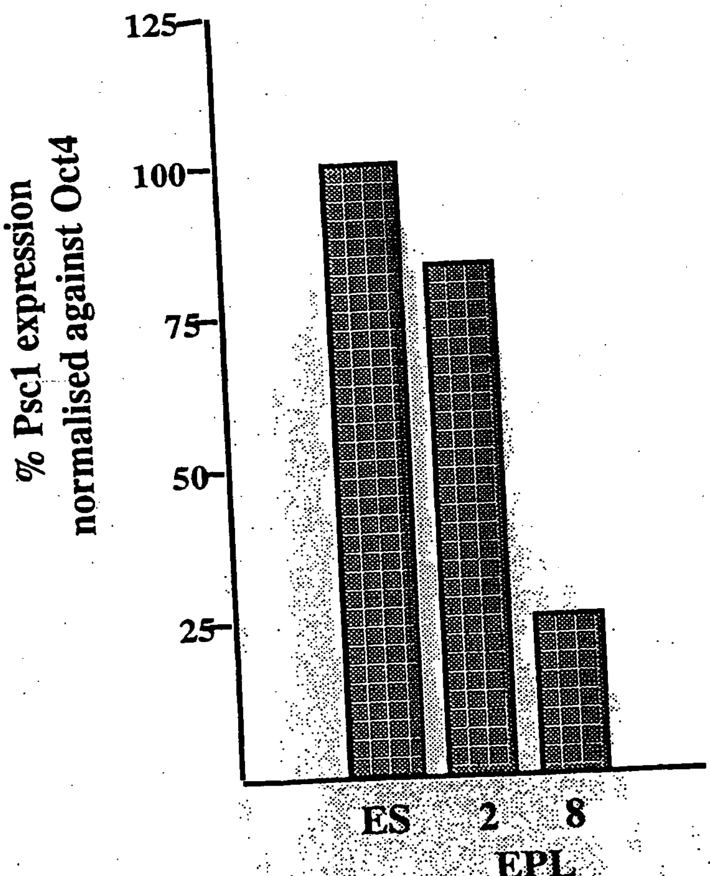
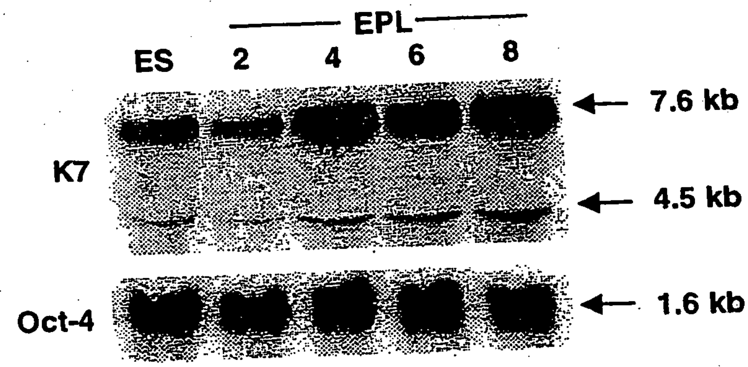
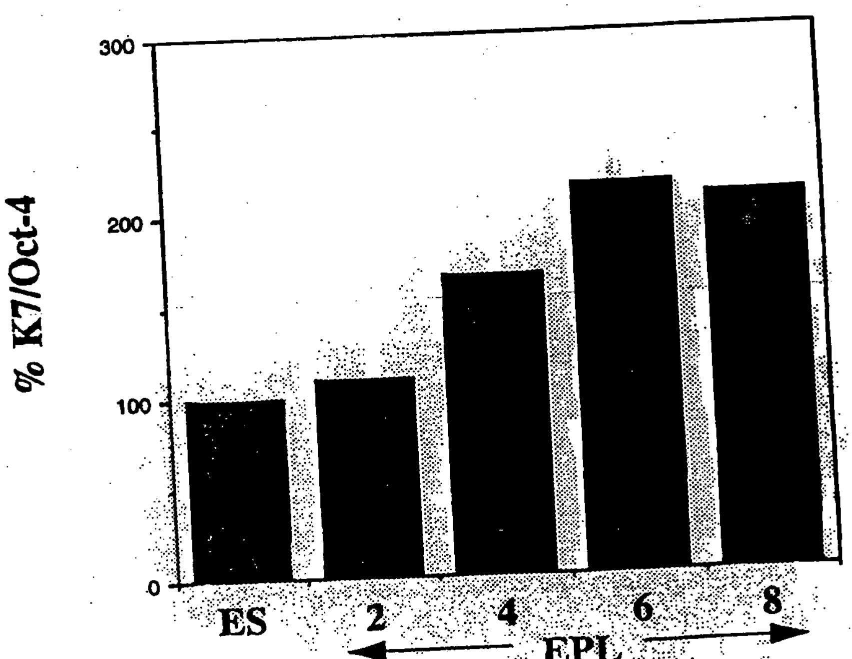

125
100
75
50
25
% Pscl expression
normalised against Oct4
ES 2 8
EPL

FIGURE 4C


EPL
ES
2
4
6
8
K7
7.6 kb
4.5 kb
Oct-4
1.6 kb


300
200
100
0
% K7/Oct-4
ES
2
4
6
8
EPL

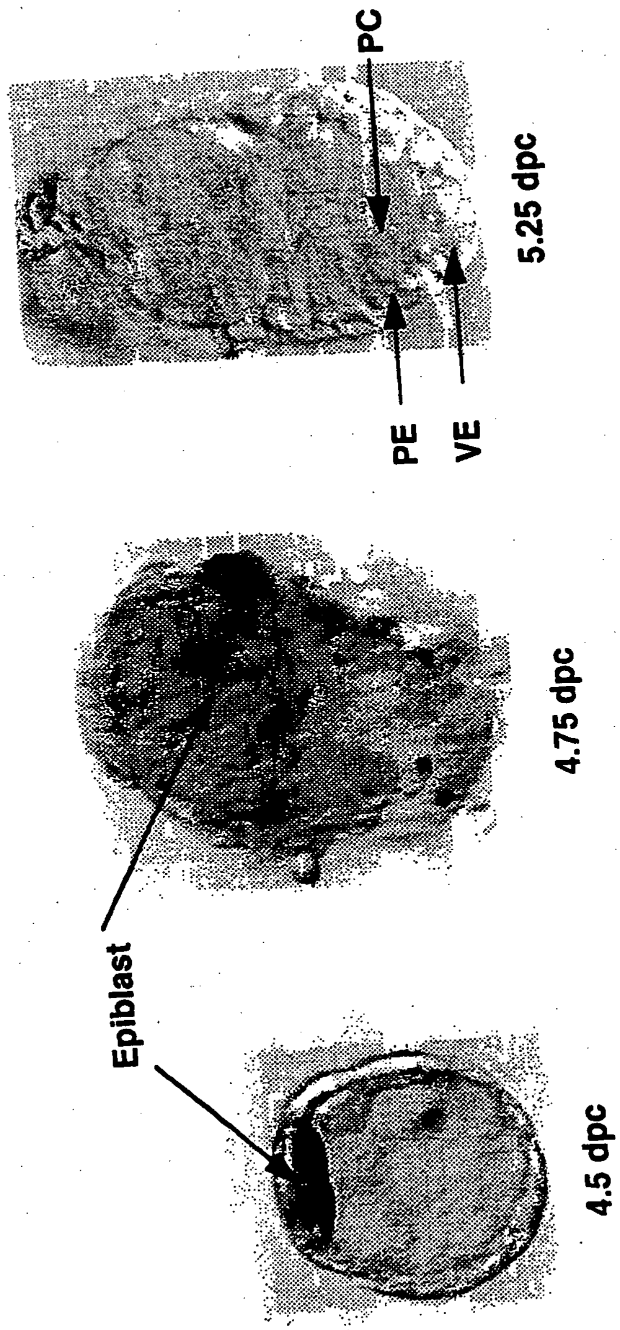


FIGURE 5A

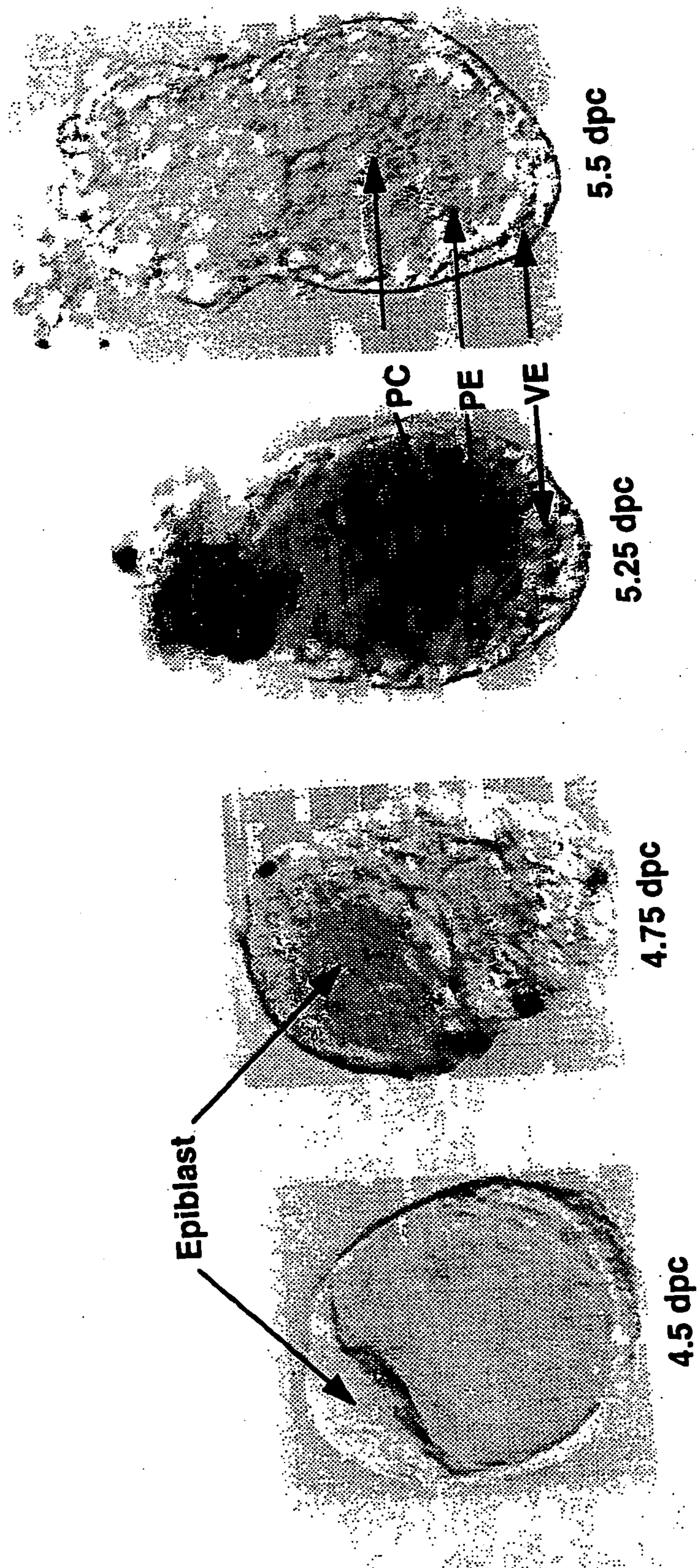


FIGURE 5B

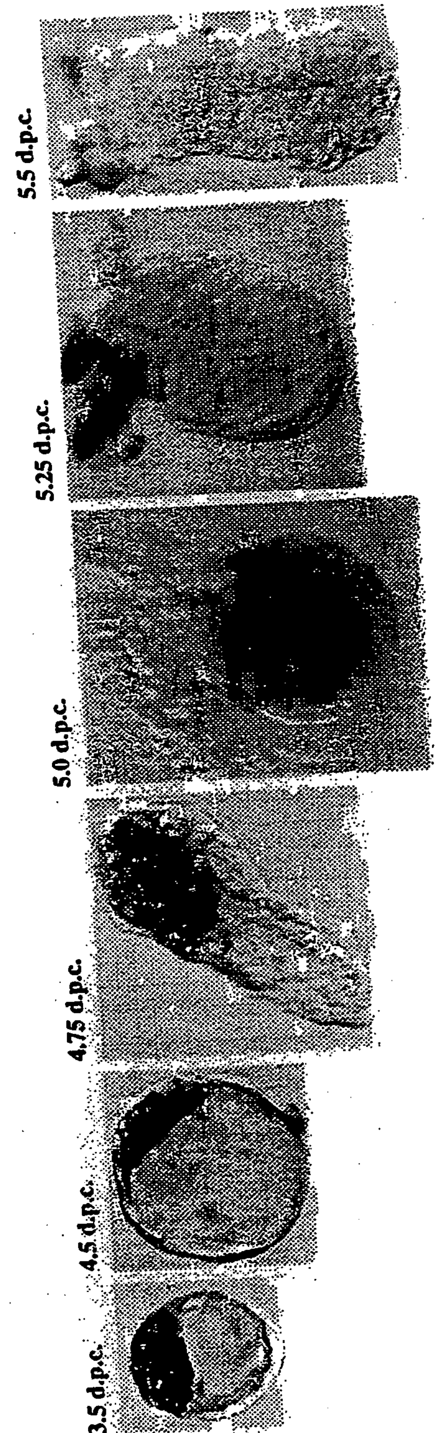


FIGURE 5C

FIGURE 6

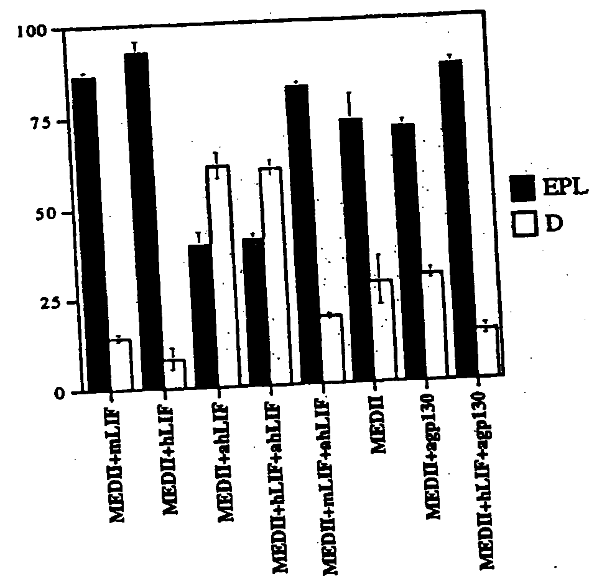

100
75
50
25
0
EPL
D
MEDII+mLIF
MEDII+hLIF
MEDII+ahLIF
MEDII+hLIF+ahLIF
MEDII+mLIF+ahLIF
MEDII
MEDII+agp130
MEDII+hLIF+agp130

FIGURE 7A

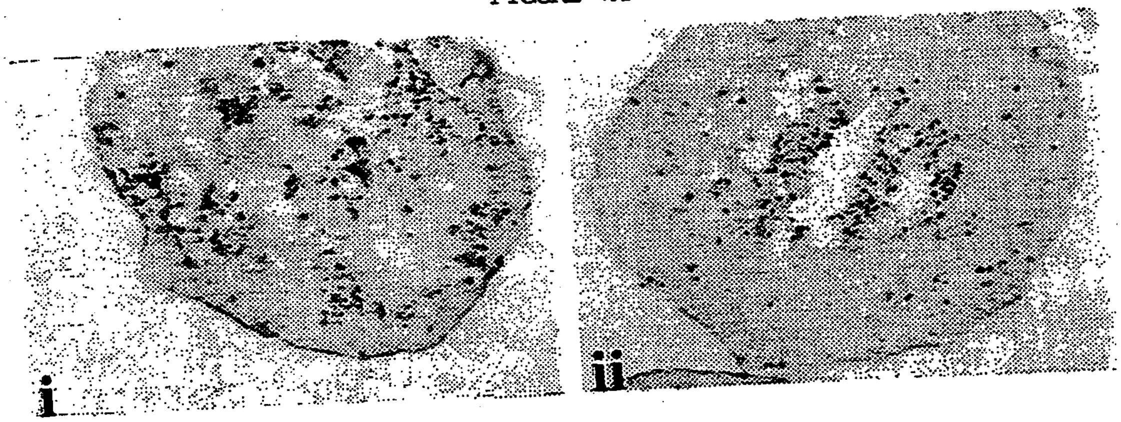


FIGURE 7B

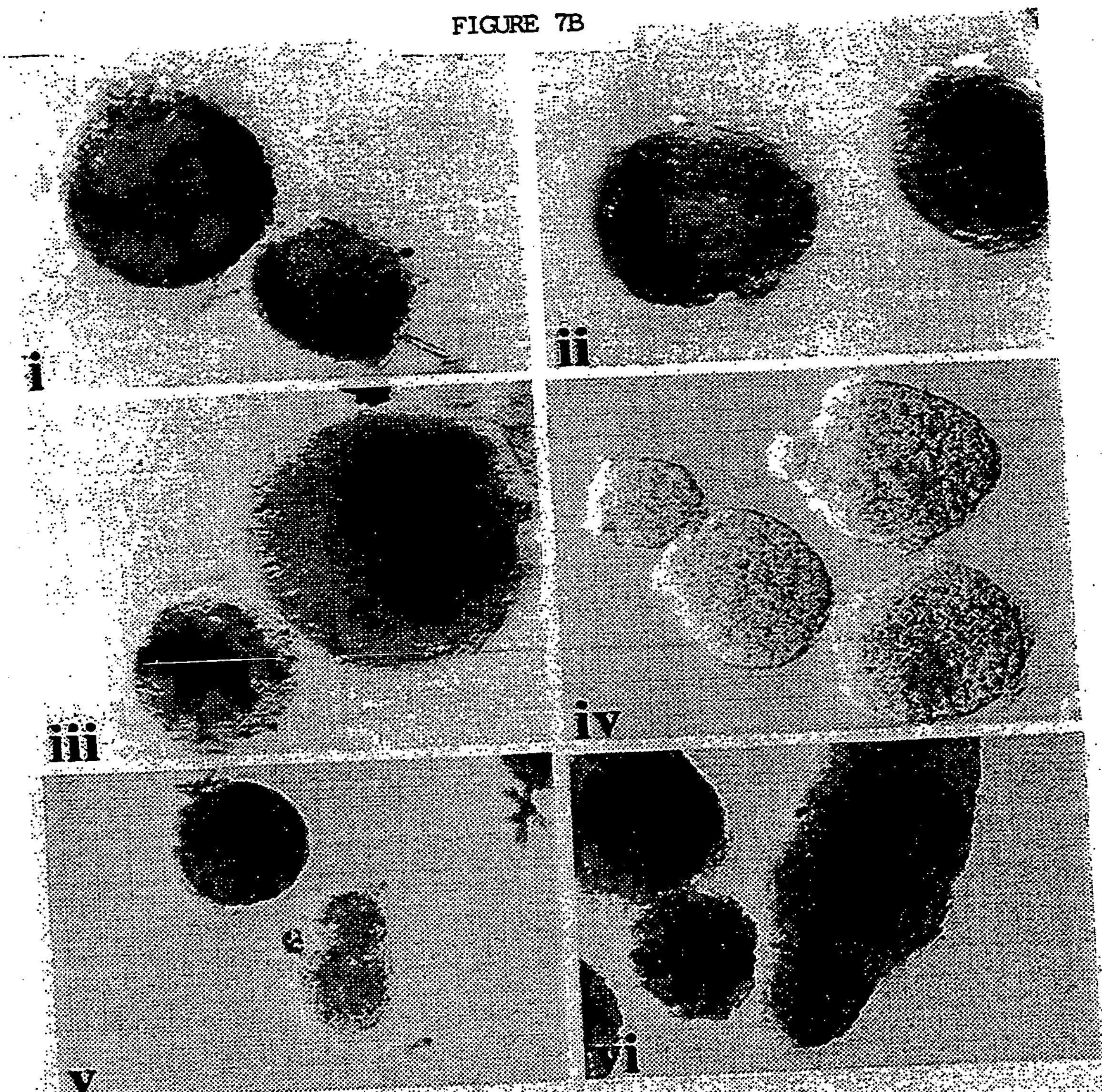


FIGURE 8

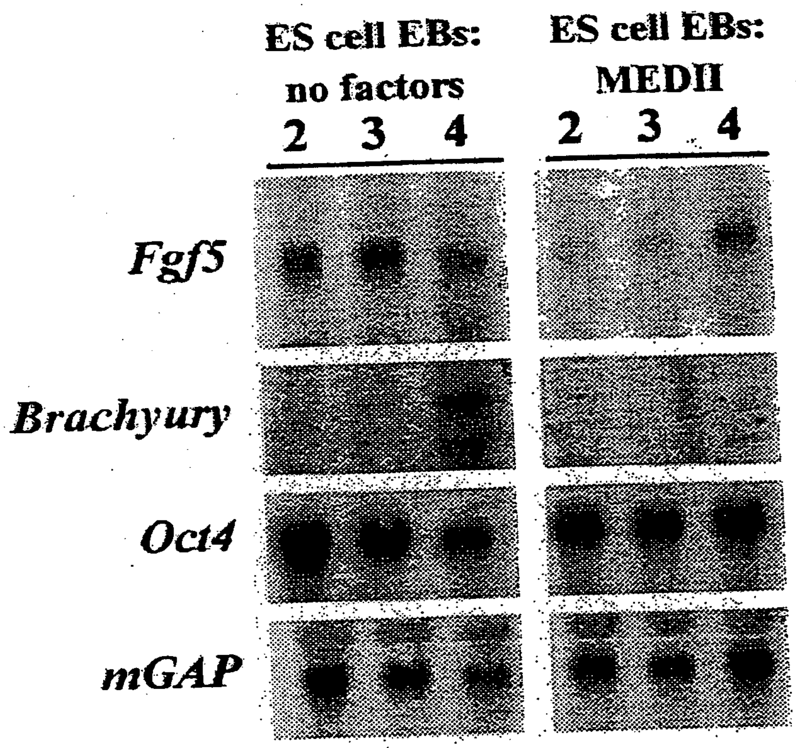

ES cell EBs:
no factors
2 3 4
ES cell EBs:
MEDII
2 3 4
Fgf5
Brachyury
Oct4
mGAP

FIGURE 9

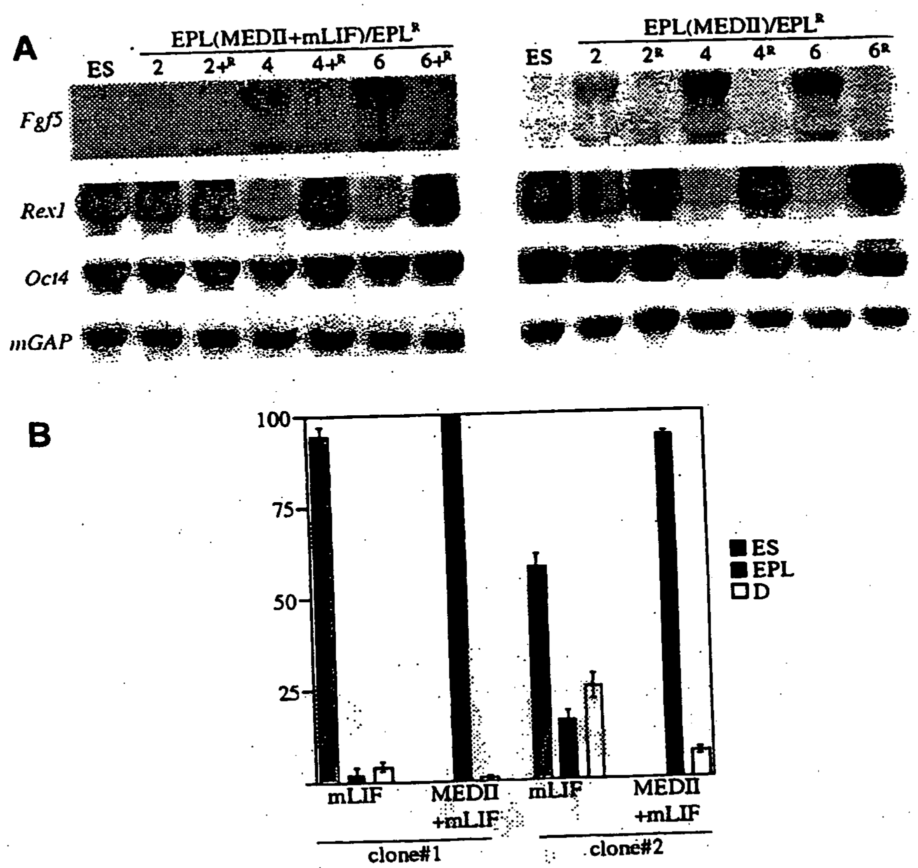

A
EPL(MEDII+mLIF)/EPL^R
ES 2 2+R 4 4+R 6 6+R
EPL(MEDII)/EPL^R
ES 2 2R 4 4R 6 6R
Fgf5
Rex1
Oct4
mGAP
B
100
75
50
25
ES
EPL
D
mLIF
MEDII +mLIF
mLIF
MEDII +mLIF
clone#1
clone#2

FIGURE 10

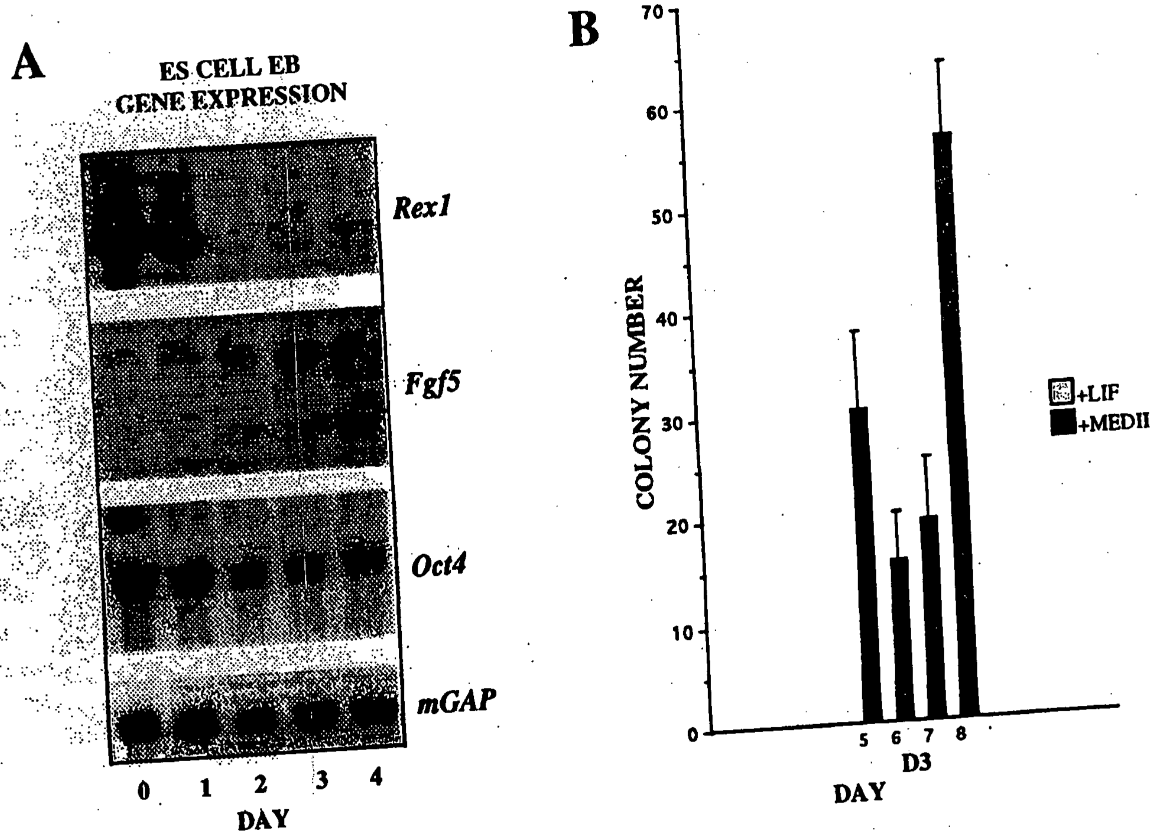

A
ES CELL EB
GENE EXPRESSION
Rex1
Fgf5
Oct4
mGAP
0 1 2 3 4
DAY
B
70
60
50
40
30
20
10
0
COLONY NUMBER
+LIF
+MEDII
5 6 7 8
D3
DAY

FIGURE II

A

B

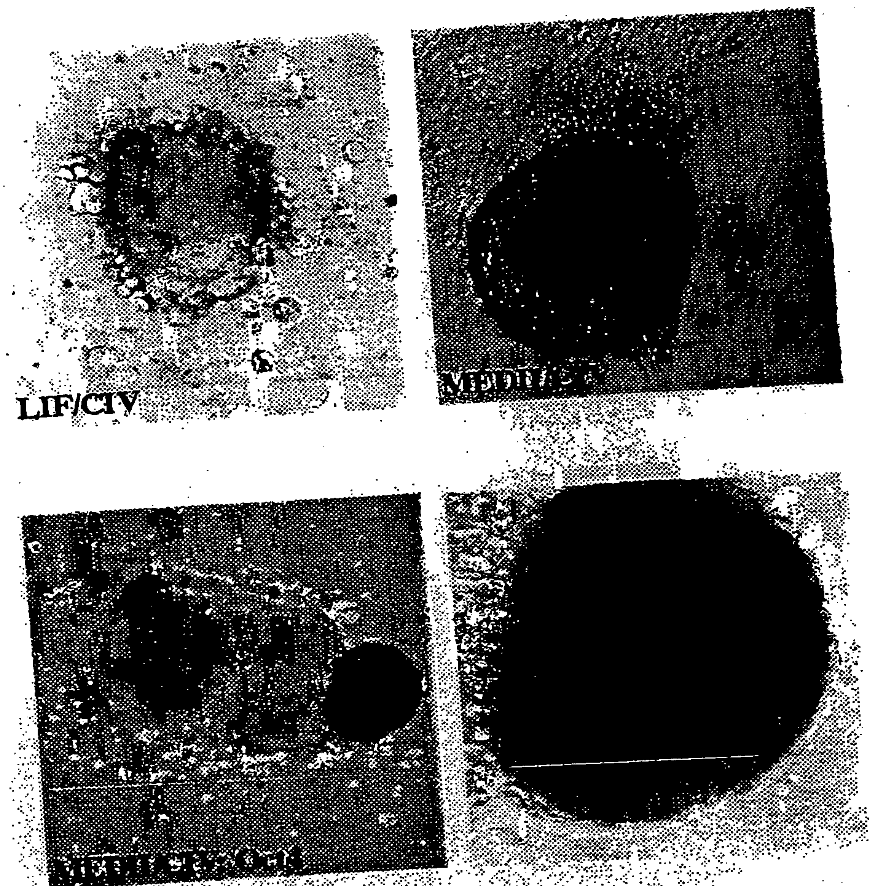
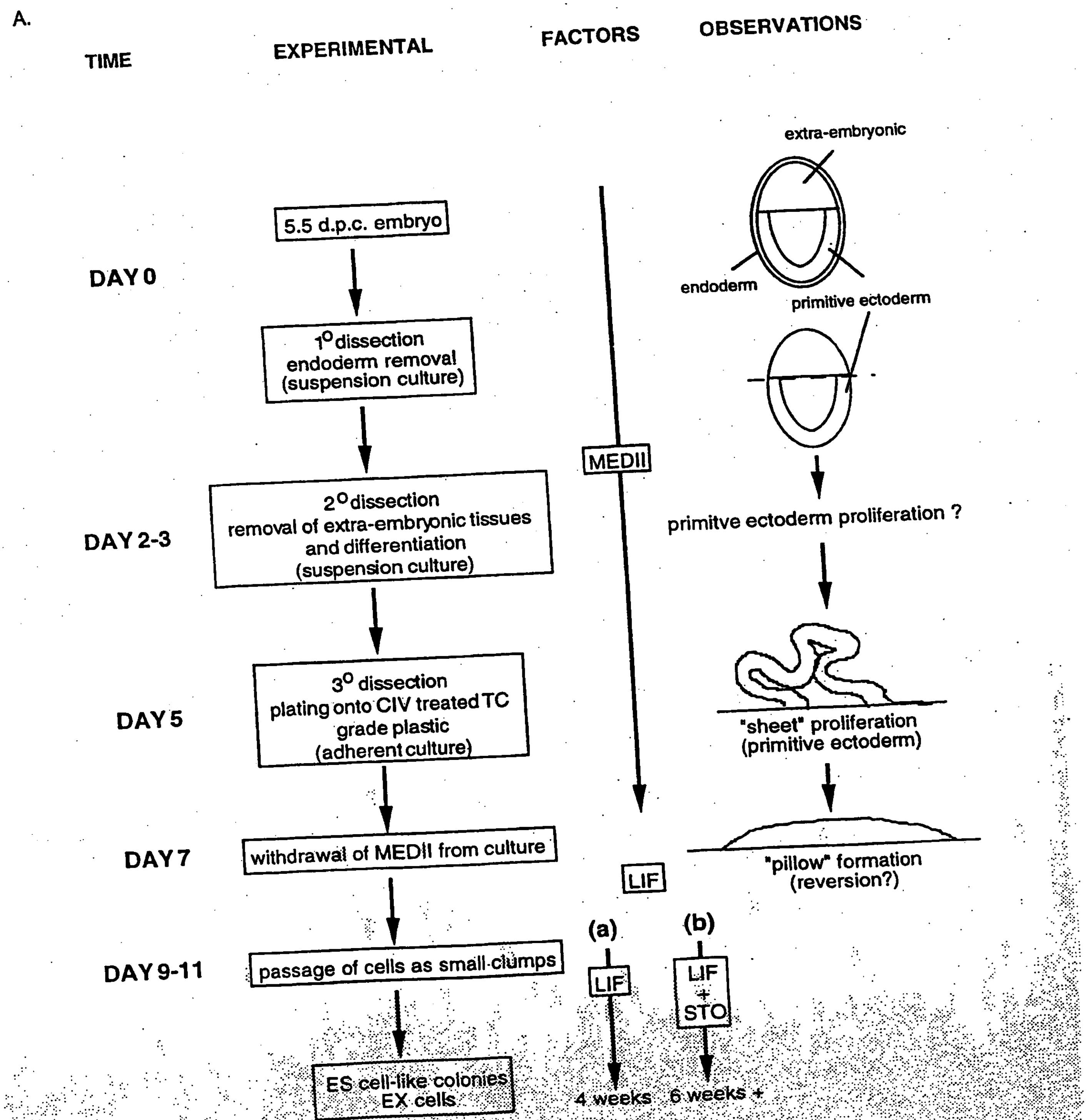


FIGURE 12A


A.
TIME
EXPERIMENTAL
FACTORS
OBSERVATIONS
extra-embryonic
5.5 d.p.c. embryo
DAY 0
endoderm
primitive ectoderm
1° dissection
endoderm removal
(suspension culture)
MEDII
2° dissection
removal of extra-embryonic tissues
and differentiation
(suspension culture)
DAY 2-3
primitve ectoderm proliferation ?
3° dissection
plating onto CIV treated TC
grade plastic
(adherent culture)
DAY 5
"sheet" proliferation
(primitive ectoderm)
DAY 7
withdrawal of MEDII from culture
LIF
"pillow" formation
(reversion?)
(a)
(b)
DAY 9-11
passage of cells as small clumps
LIF
LIF
+
STO
ES cell-like colonies
EX cells
4 weeks
6 weeks +

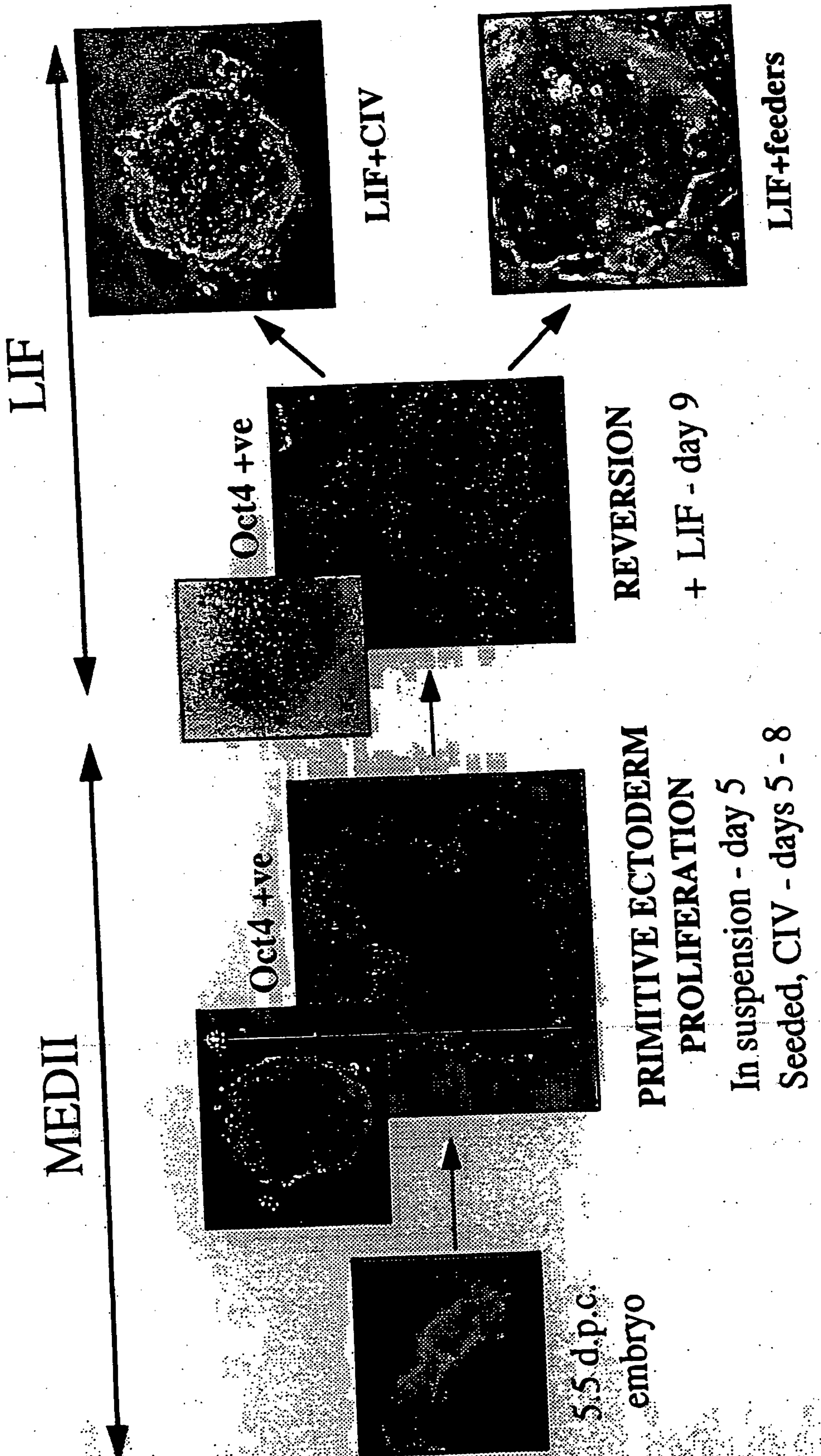
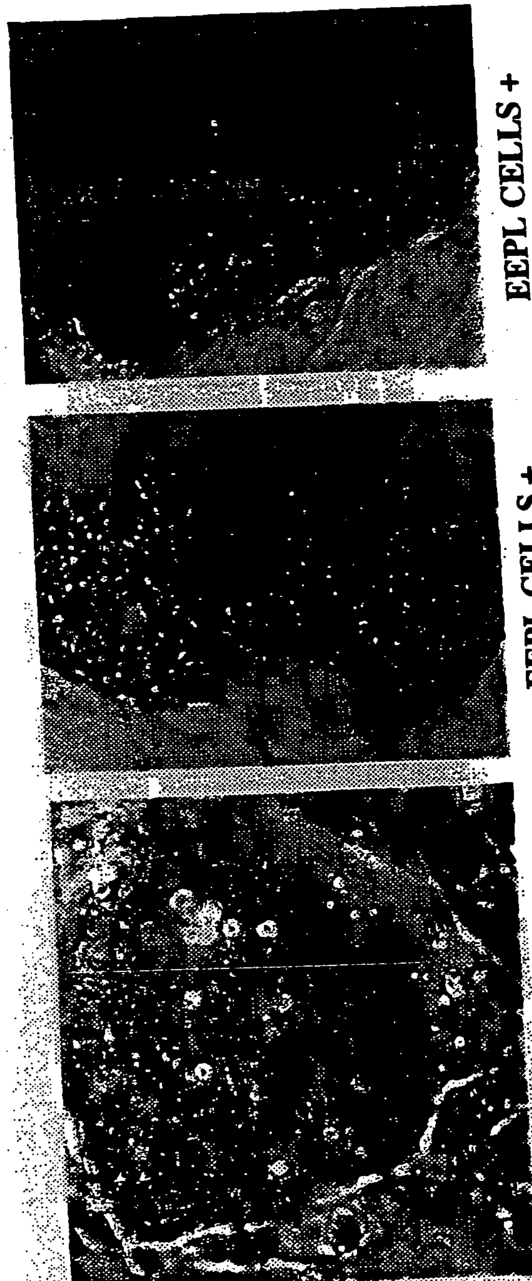

LIF
MEDII
LIF+CIV
LIF+feeders
Oct4 +ve
REVERSION
+ LIF - day 9
Oct4 +ve
PRIMITIVE ECTODERM
PROLIFERATION
In suspension - day 5
Seeded, CIV - days 5 - 8
5.5 d.p.c.
embryo

FIGURE 12B



EEPL CELLS + FEEDERS Oct-4

EEPL CELLS + FEEDERS Alkaline phosphatase

EEPL CELLS + FEEDERS

FIGURE 13

FIGURE 14

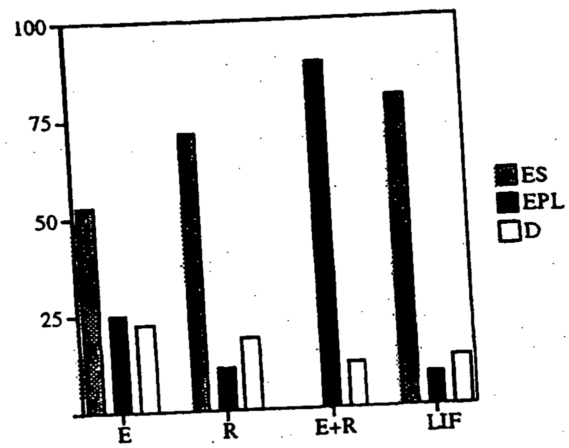


FIGURE 15

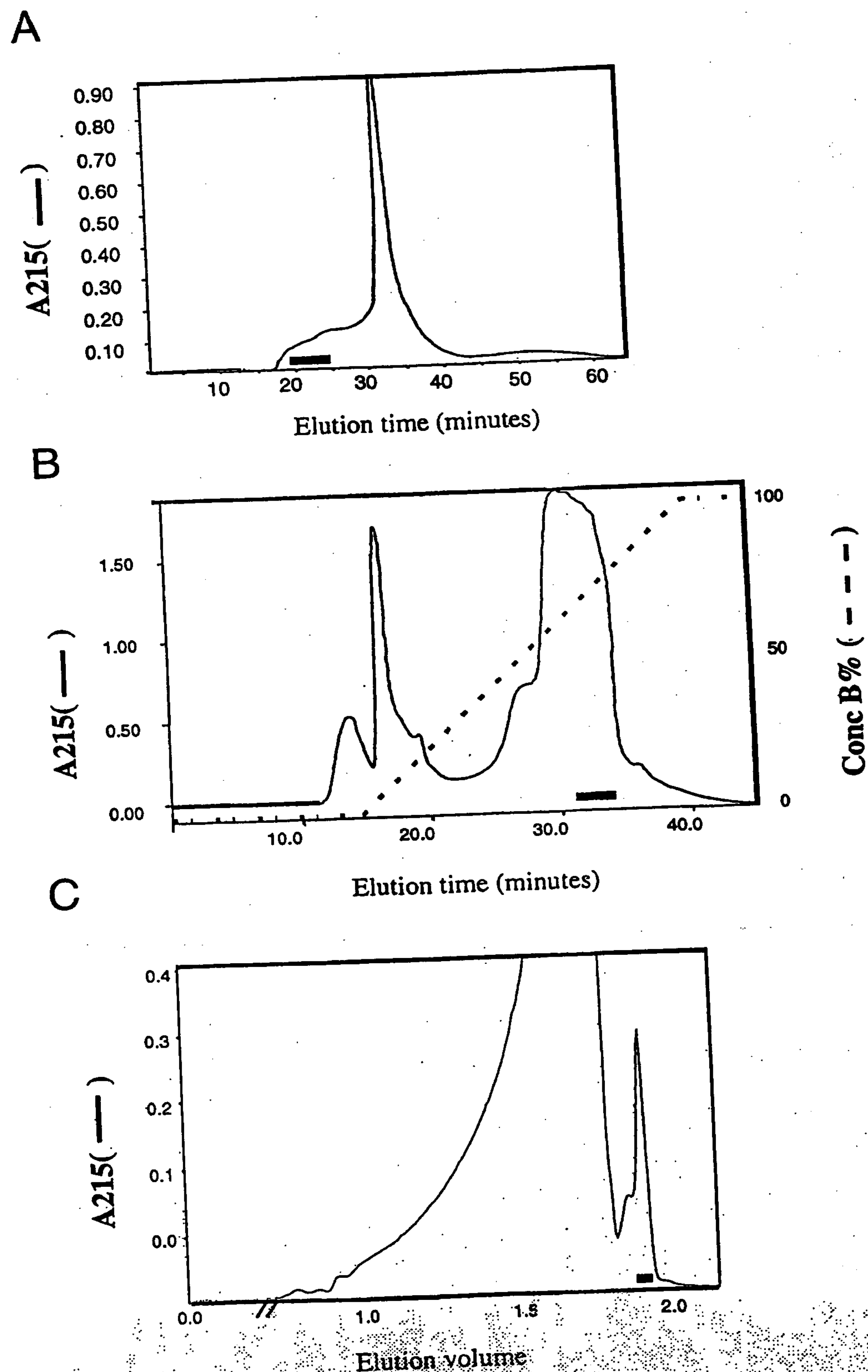
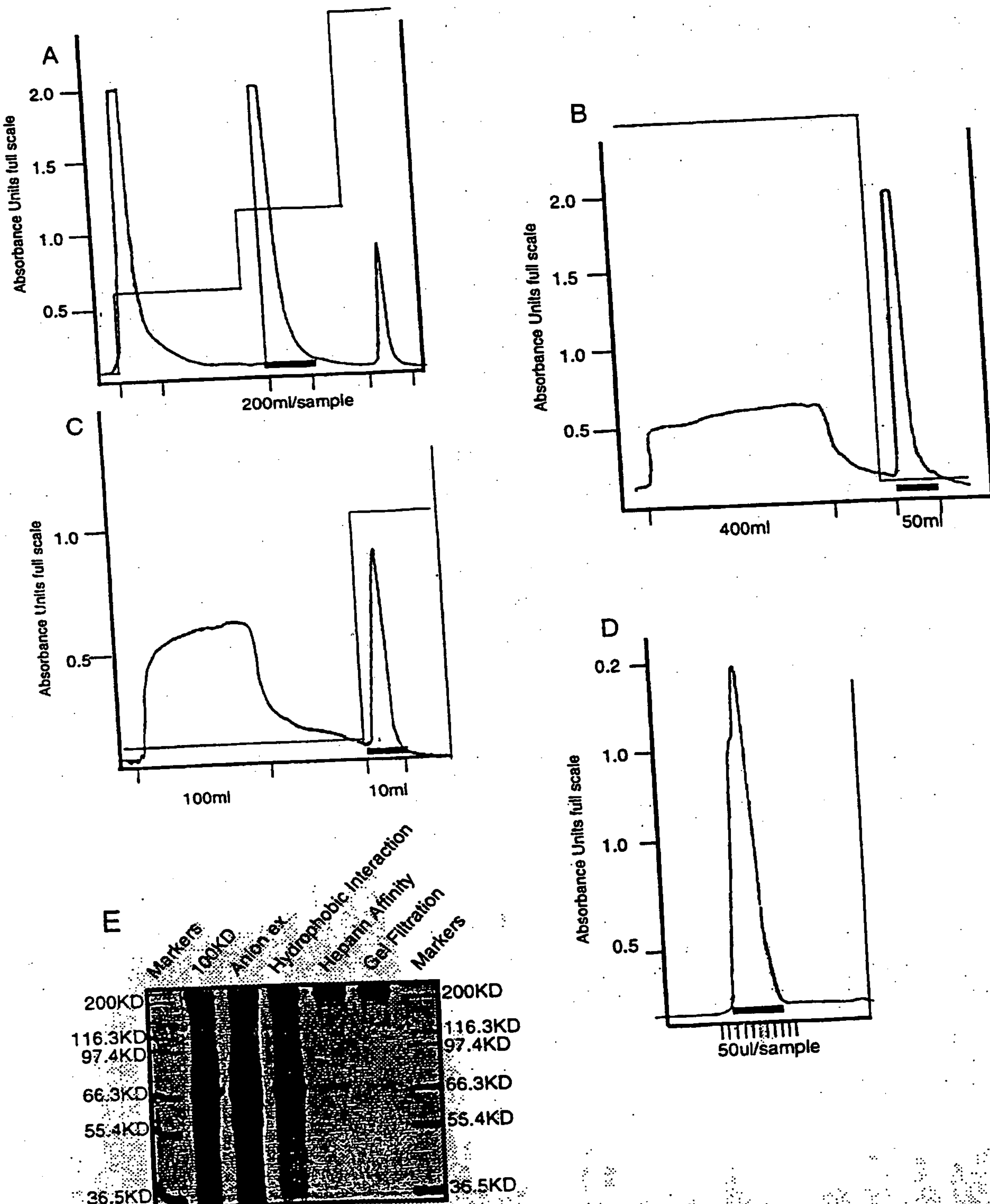

A
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
A215(—)
10
20
30
40
50
60
Elution time (minutes)
B
1.50
1.00
0.50
0.00
A215(—)
100
50
0
Conc B% (- - -)
10.0
20.0
30.0
40.0
Elution time (minutes)
C
0.4
0.3
0.2
0.1
0.0
A215(—)
0.0
1.0
1.5
2.0
Elution volume

FIGURE 16


A
2.0
1.5
1.0
0.5
Absorbance Units full scale
200ml/sample
B
2.0
1.5
1.0
0.5
Absorbance Units full scale
400ml
50ml
C
1.0
0.5
Absorbance Units full scale
100ml
10ml
D
0.2
1.0
1.0
0.5
Absorbance Units full scale
50ul/sample
E
Markers
100KD
Anion ex
Hydrophobic Interaction
Heparin Affinity
Gel Filtration
Markers
200KD
116.3KD
97.4KD
66.3KD
55.4KD
36.5KD
200KD
116.3KD
97.4KD
66.3KD
55.4KD
36.5KD

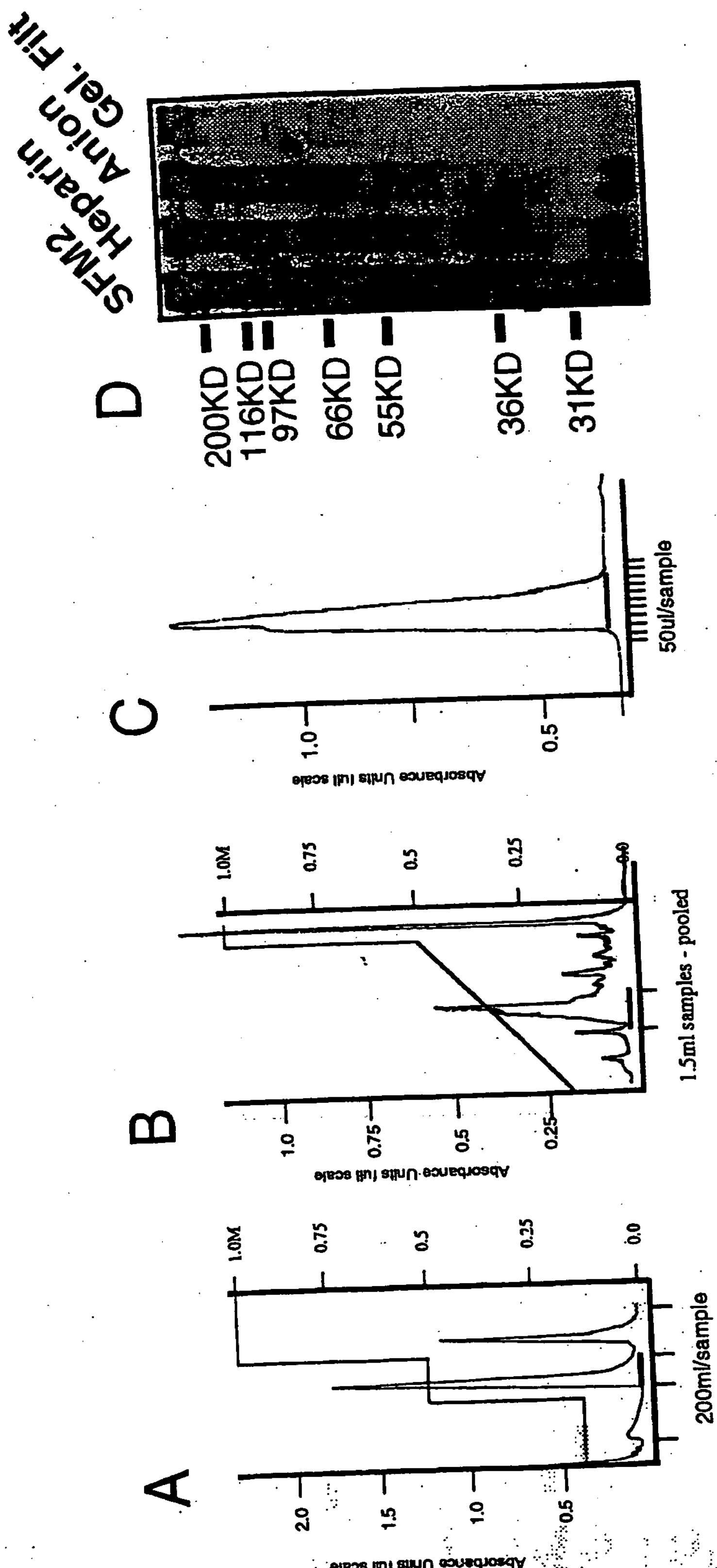
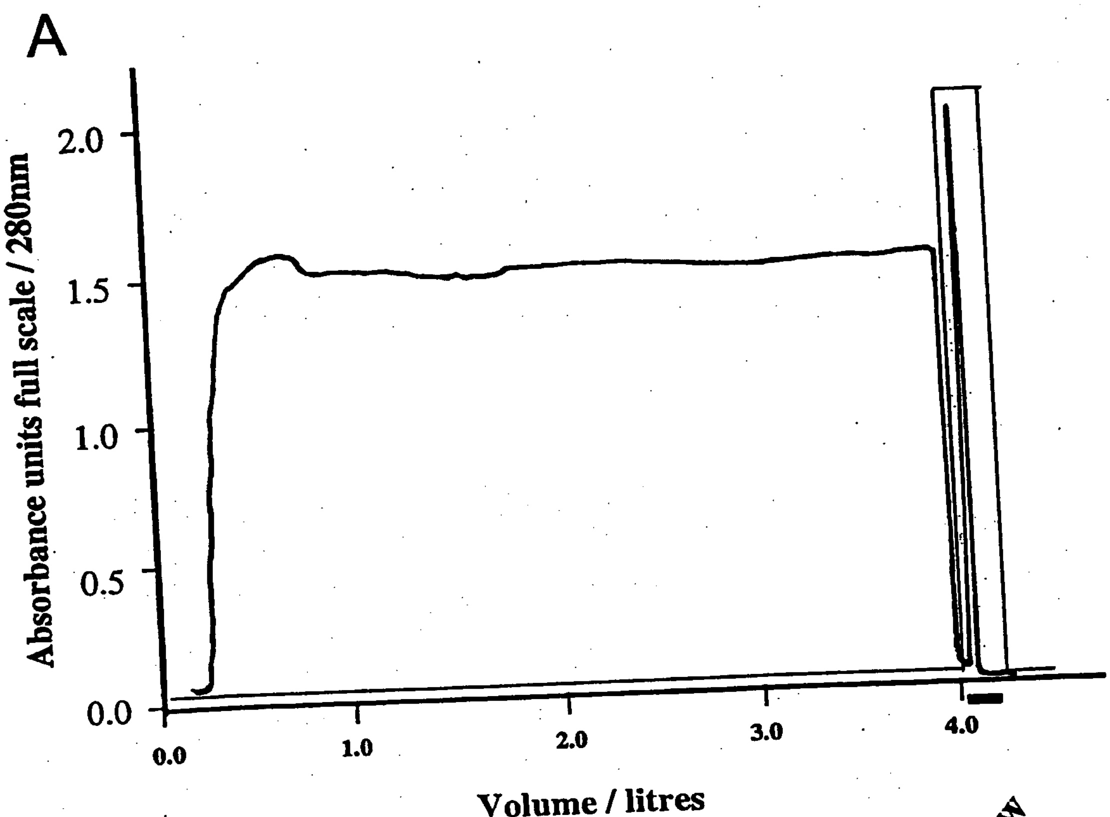

A
Absorbance Units (full scale)
2.0
1.5
1.0
0.5
1.0M
0.75
0.5
0.25
0.0
200ml/sample
B
Absorbance Units (full scale)
1.0
0.75
0.5
0.25
1.0M
0.75
0.5
0.25
0.0
1.5ml samples - pooled
C
Absorbance Units (full scale)
1.0
0.5
50ul/sample
D
200KD
116KD
97KD
66KD
55KD
36KD
31KD
SFM2
Heparin
Anion
Gel. Filt

FIGURE 17


A
Absorbance units full scale / 280nm
2.0
1.5
1.0
0.5
0.0
0.0
1.0
2.0
3.0
4.0
Volume / litres

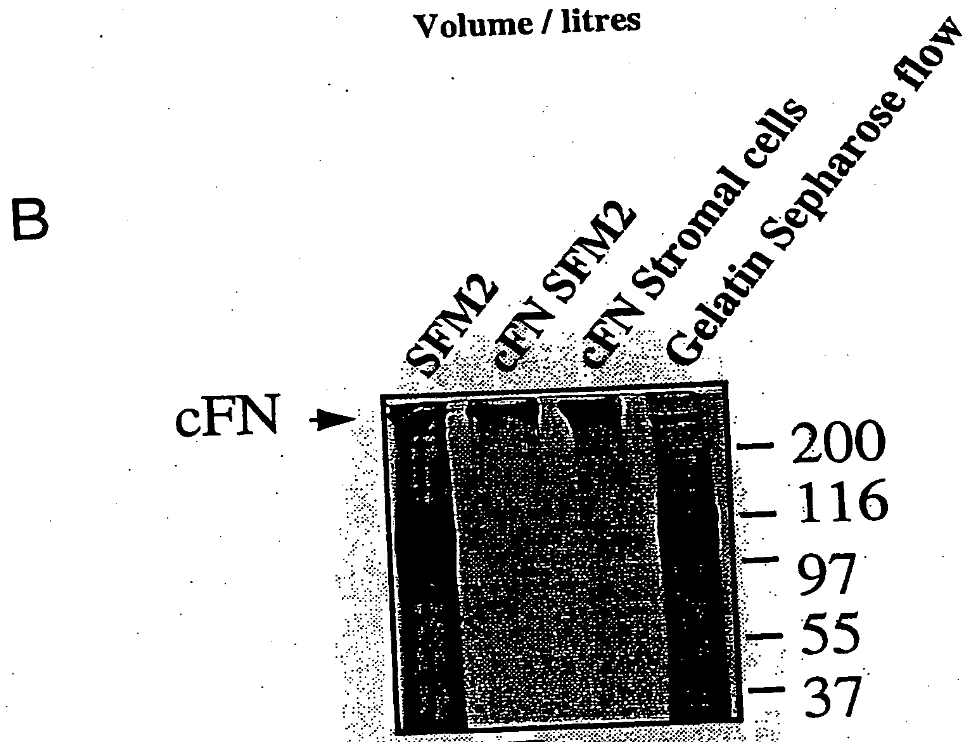

B
SFM2
cFN SFM2
cFN Stromal cells
Gelatin Sepharose flow
cFN
200
116
97
55
37

FIGURE 18


A
plasma fibronectin
+ L-proline
B
cellular fibronectin
+ L-proline
C
High Mw factor
+ L-proline

FIGURE 19

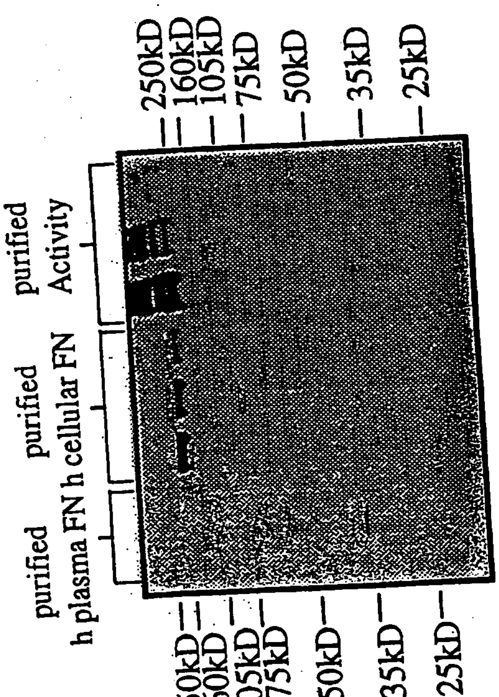

purified h plasma FN
purified h cellular FN
purified Activity
250kD
160kD
105kD
75kD
50kD
35kD
25kD

FIGURE 20

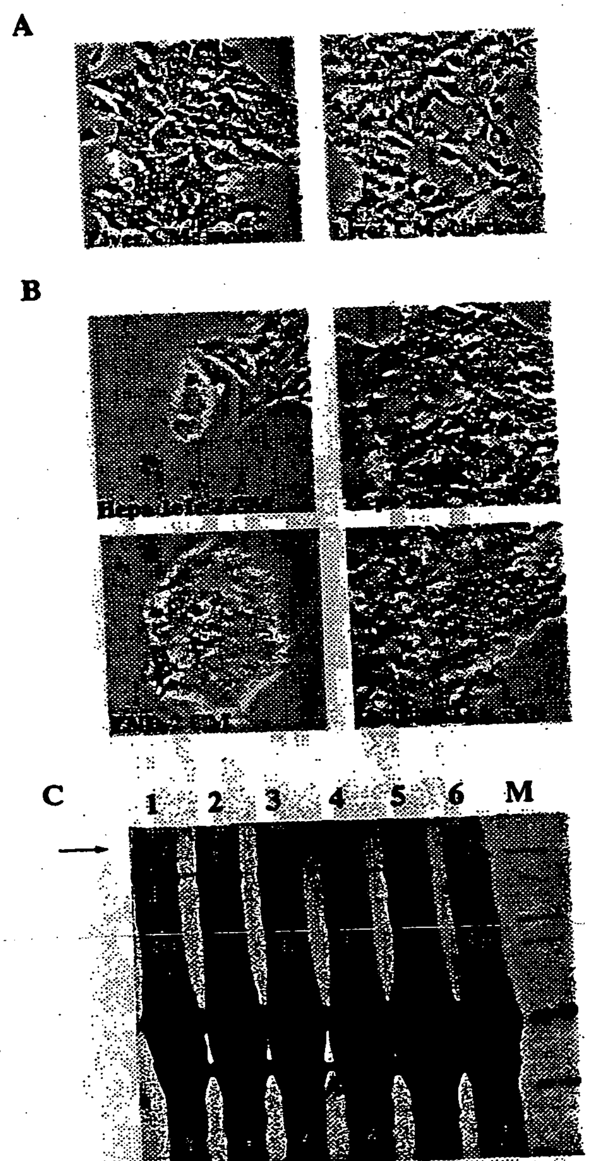


FIGURE 21

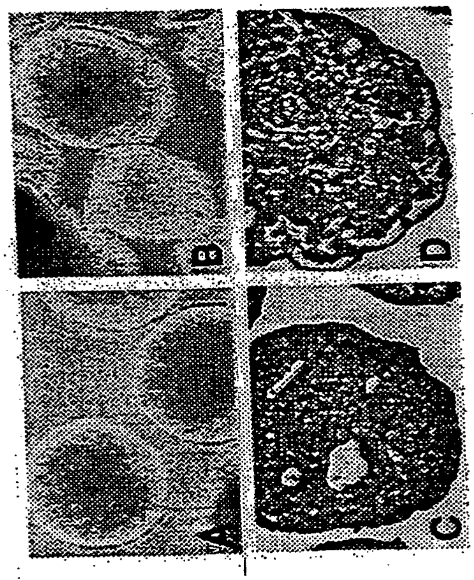


FIGURE 22

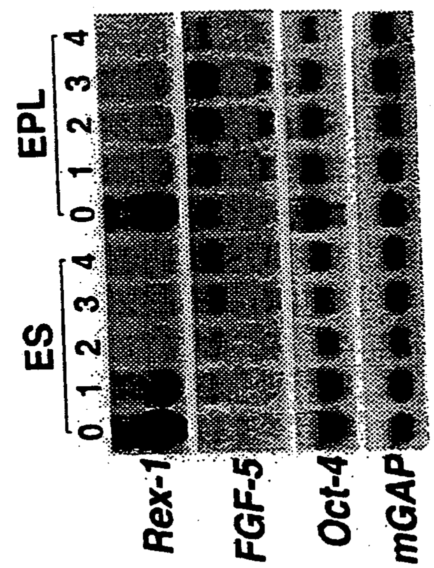


FIGURE 23

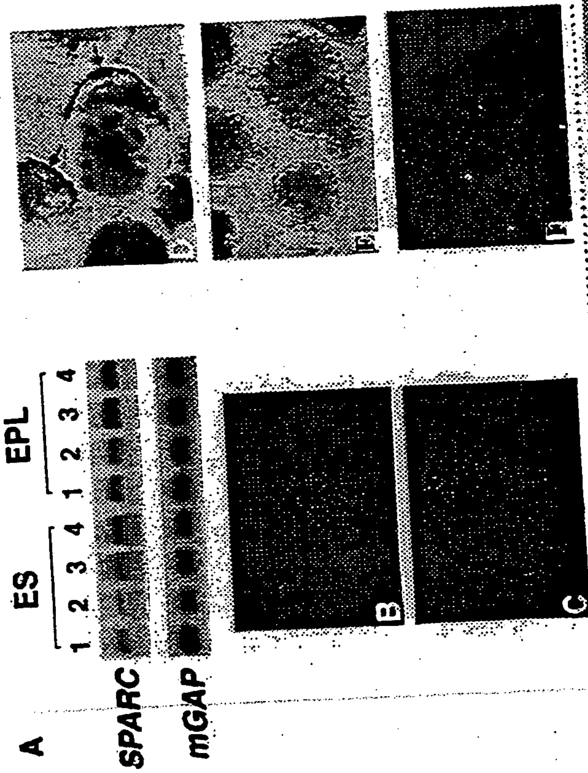


FIGURE 24

FIGURE 25

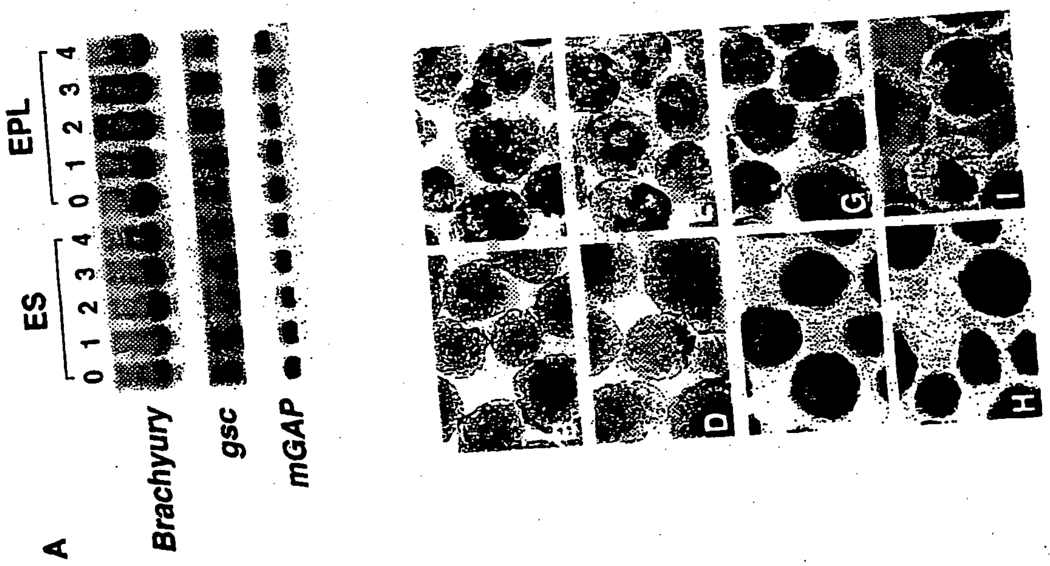
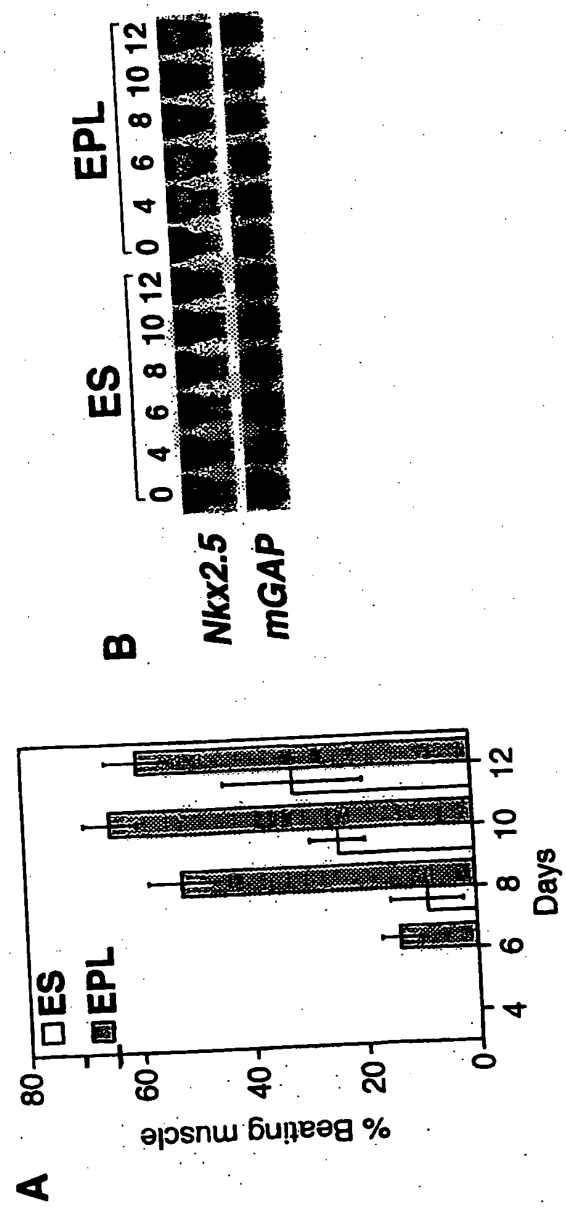


FIGURE 26

FIGURE 27


A
% Beating muscle
ES
EPL
Days
B
Nkx2.5
mGAP
ES
EPL
0 4 6 8 10 12
0 4 6 8 10 12

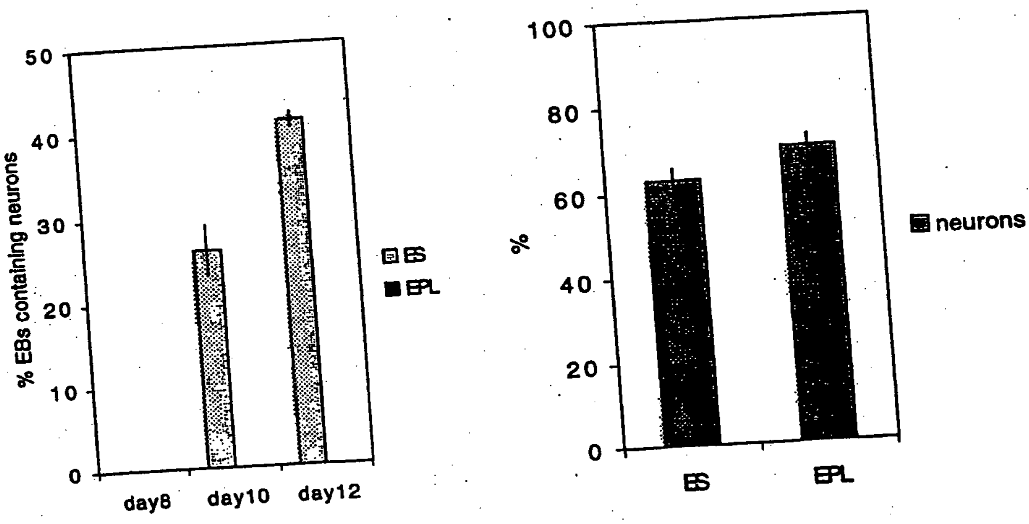

50
40
30
20
10
0
% EBs containing neurons
day8
day10
day12
ES
EPL
100
80
60
40
20
0
%
ES
EPL
neurons

FIGURE 28

FIGURE 29A

FIGURE 29C

FIGURE 29B

CONDITIONS	% COLONIES				PLATING EFFICIENCY (%)
	ES	X	SD	D	
ES	81.1+/-1.4	7.2+/-1.2	3.9+/-0.8	7.8+/-1.3	35.2+/-4.1
ES + bFGF	80.6+/-2.5	4.4+/-0.6	5.2+/-1.4	9.8+/-1.2	36.2+/-2.4
X	0	58.3+/-6.1	31.3+/-3.7	10.4+/-3.1	42.5+/-3.2
X + bFGF	0	21.0+/-3.1	45.2+/-2.4	33.9+/-0.8	42.0+/-1.6

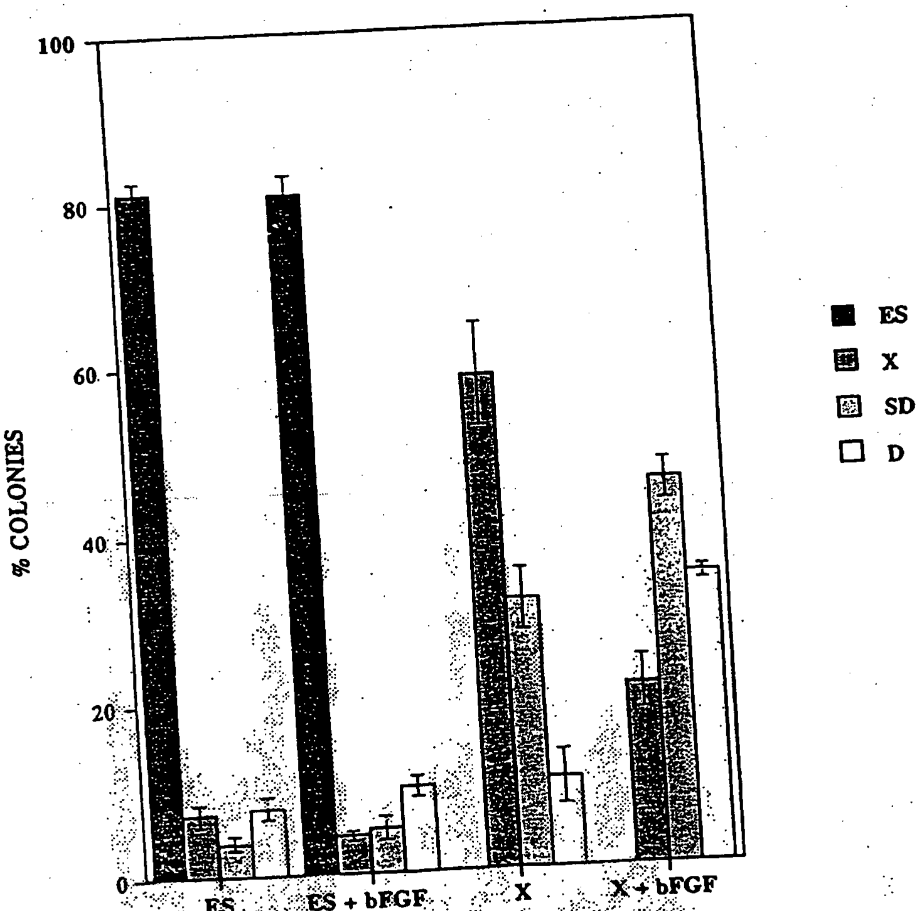
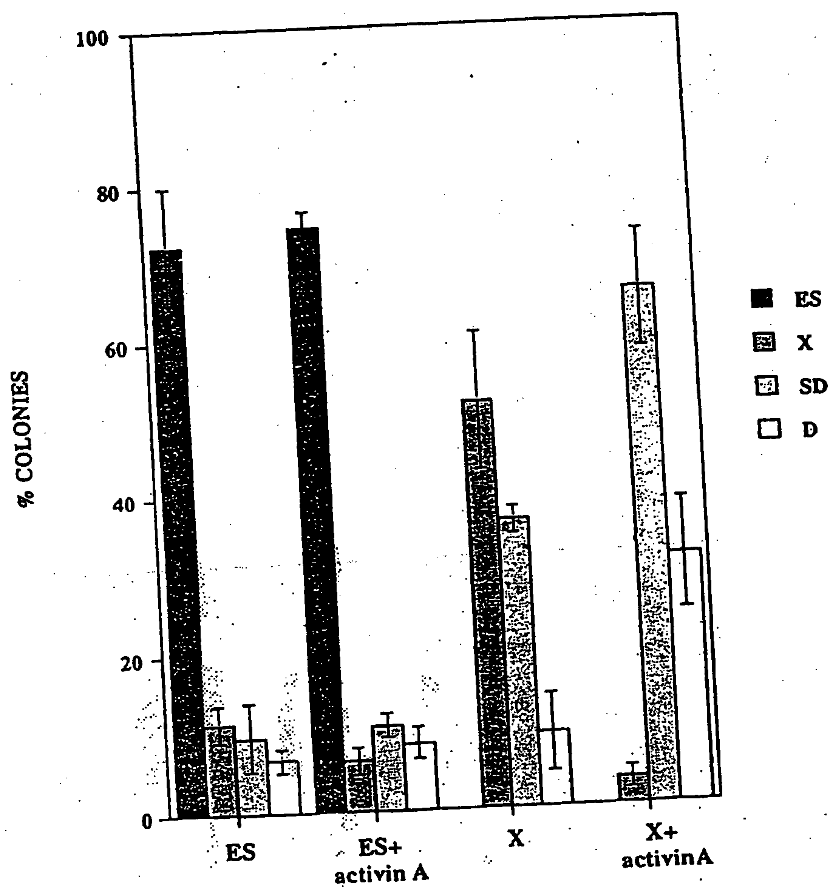


FIGURE 29D

CONDITIONS	% COLONIES				PLATING EFFICIENCY (%)
	ES	X	SD	D	
ES	72.5+/-7.5	11.3+/-2.3	9.6+/-4.2	6.6+/-1.5	33.6+/-1.6
ES + activin A	74.5+/-2.1	6.4+/-2.1	10.7+/-1.5	8.4+/-2.0	35.1+/-2.3
X	0	51.2+/-8.7	36.3+/-1.7	9.2+/-4.9	39.9+/-5.4
X + activn A	0	3.28+/-1.4	65.4+/-7.5	31.3+/-7.1	41.13+/-3.8



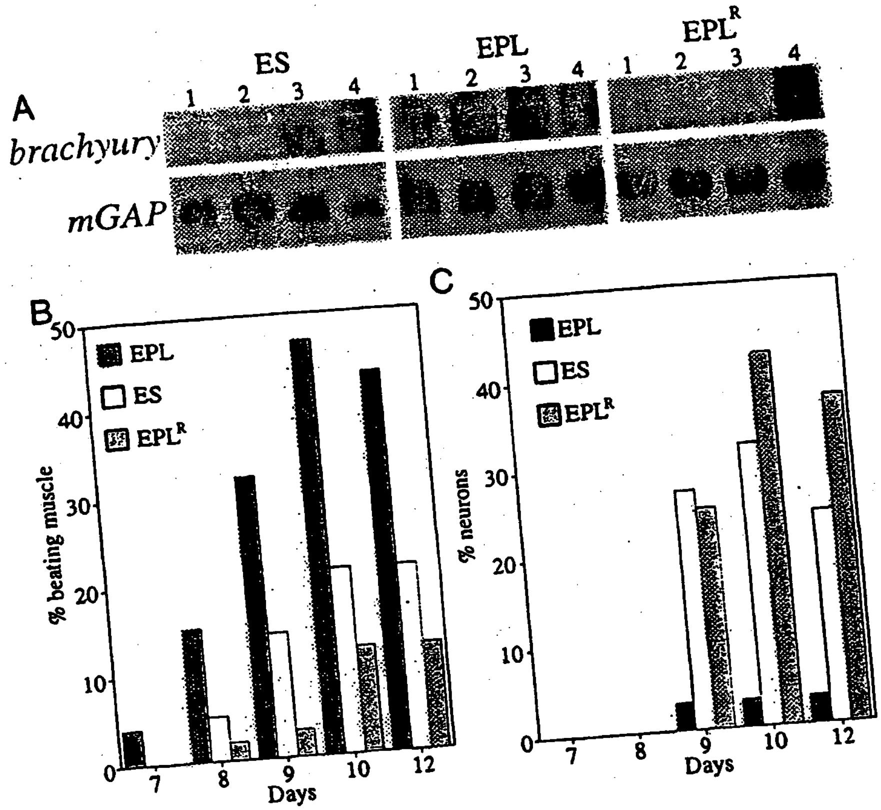

A
ES
EPL
EPL^R
1
2
3
4
brachyury
mGAP
B
% beating muscle
EPL
ES
EPL^R
Days
C
% neurons
EPL
ES
EPL^R
Days

FIGURE 30

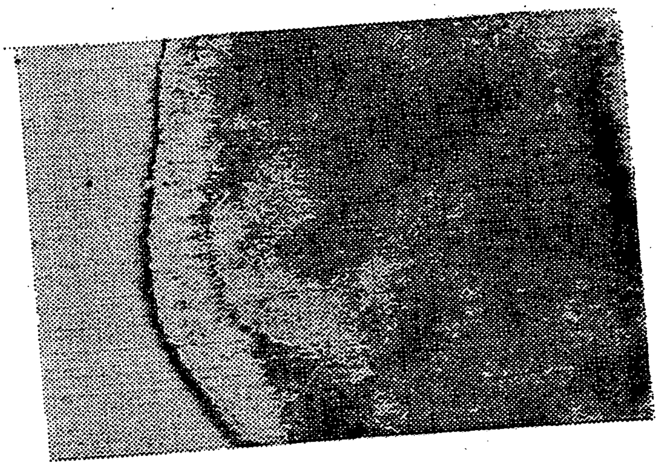


FIGURE 31A

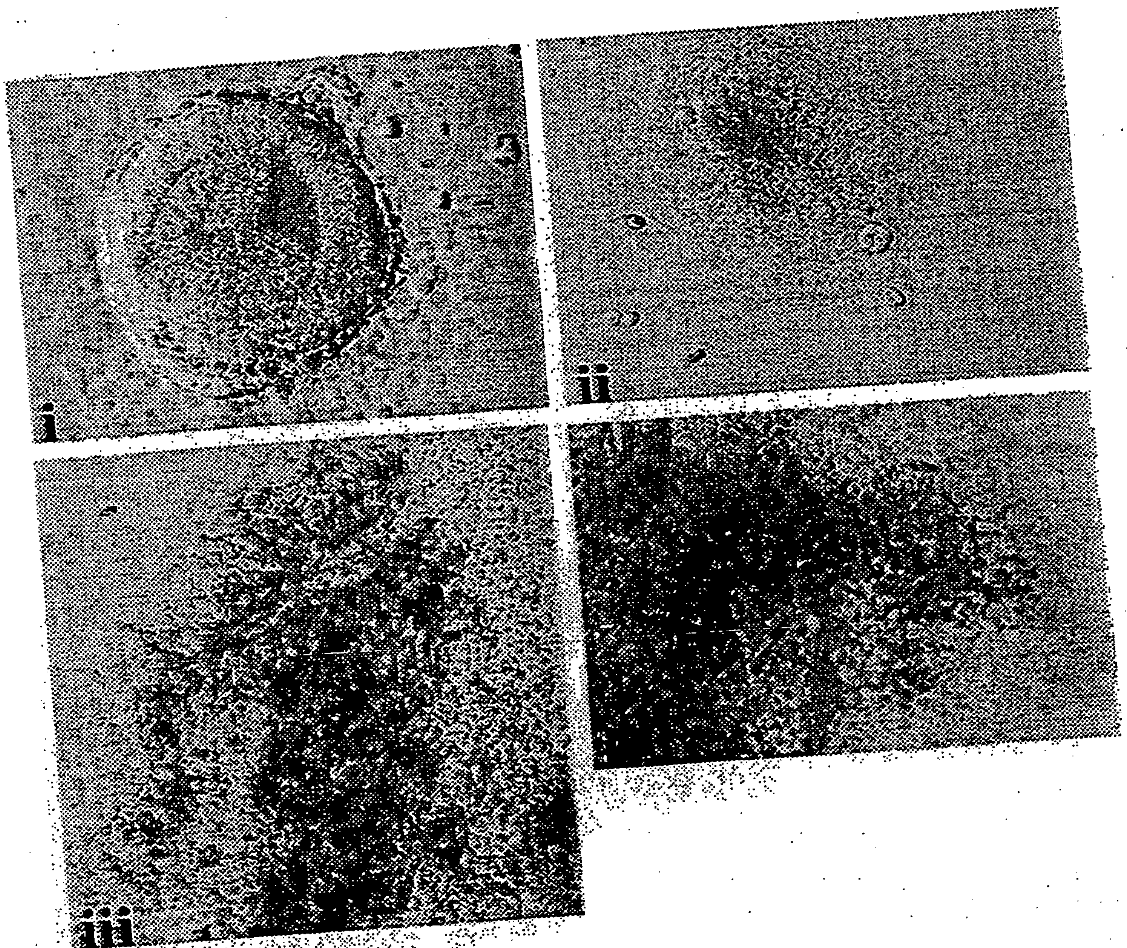


FIGURE 31B

FIGURE 32A

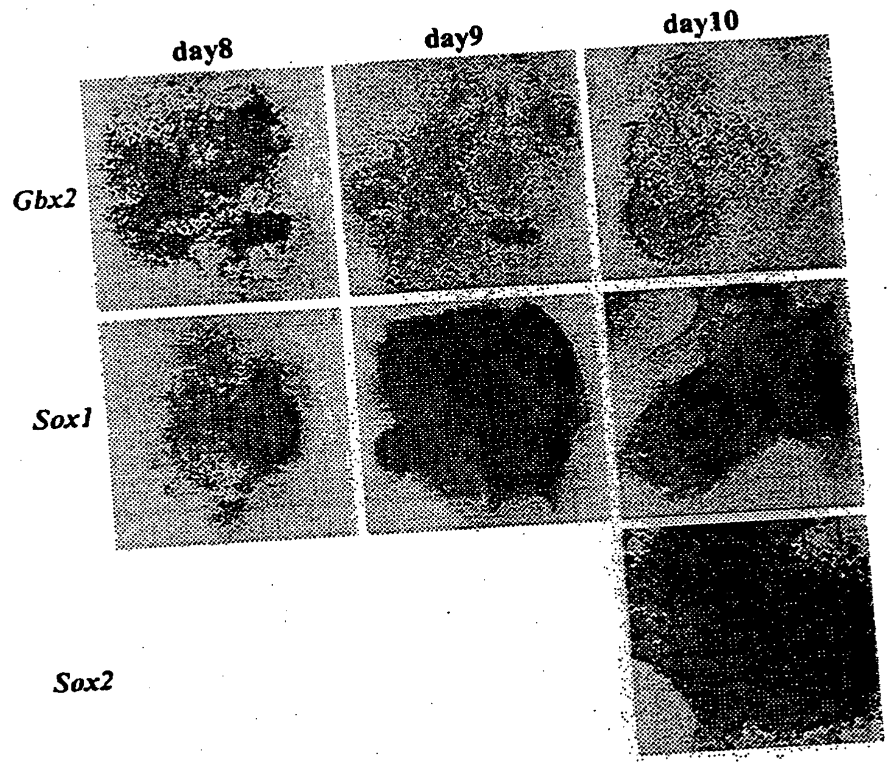
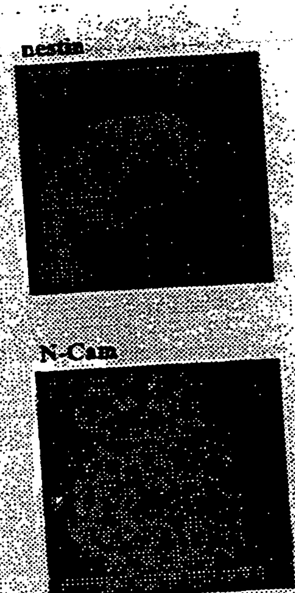


FIGURE 32B



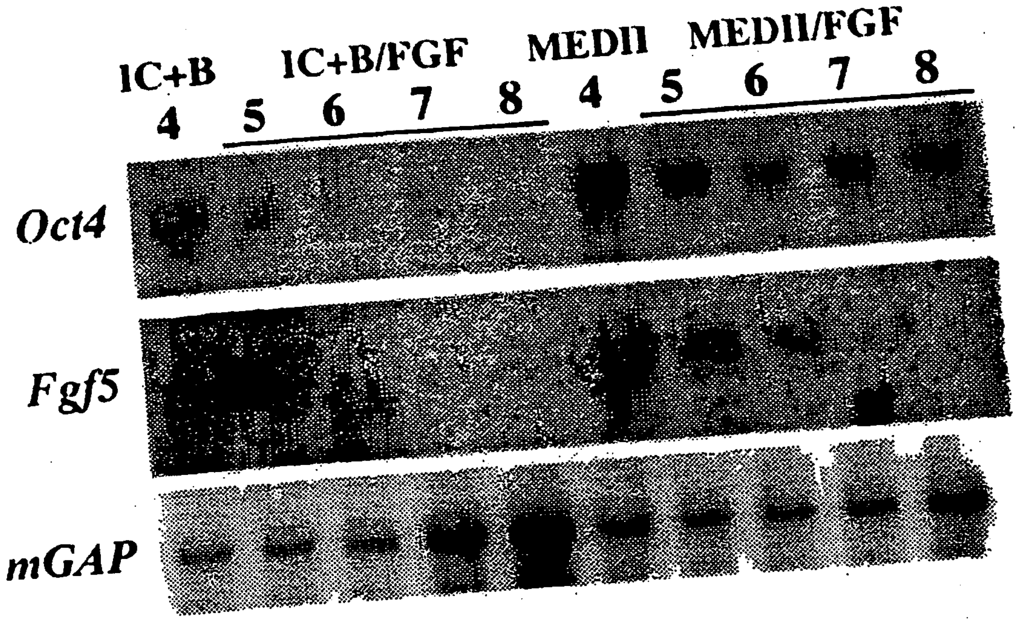


FIGURE 33A

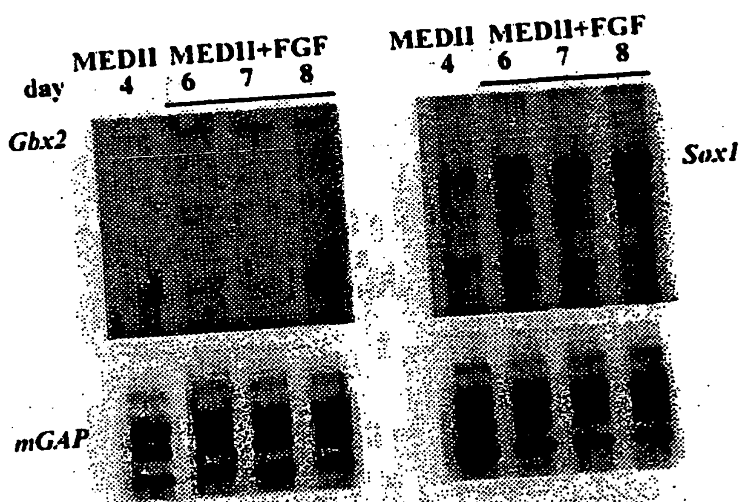


FIGURE 33B

FIGURE 34A

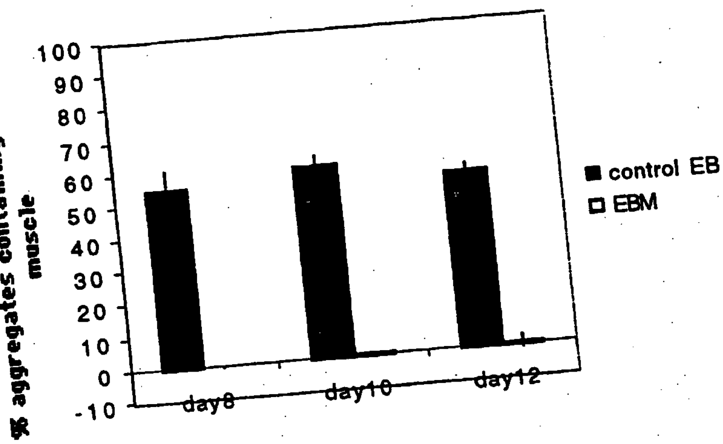


FIGURE 34B

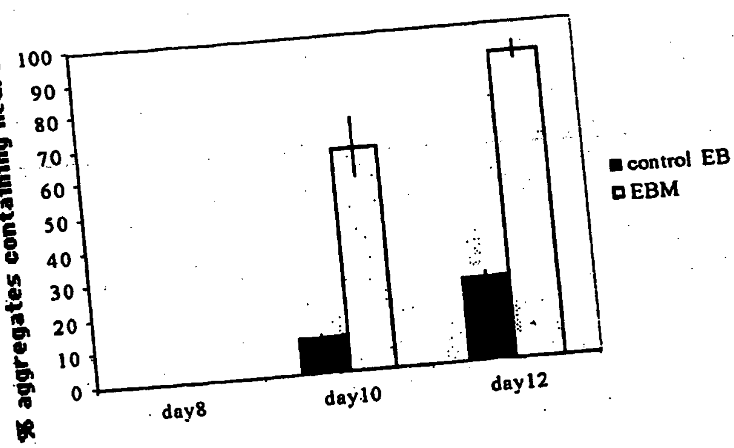


FIGURE 35

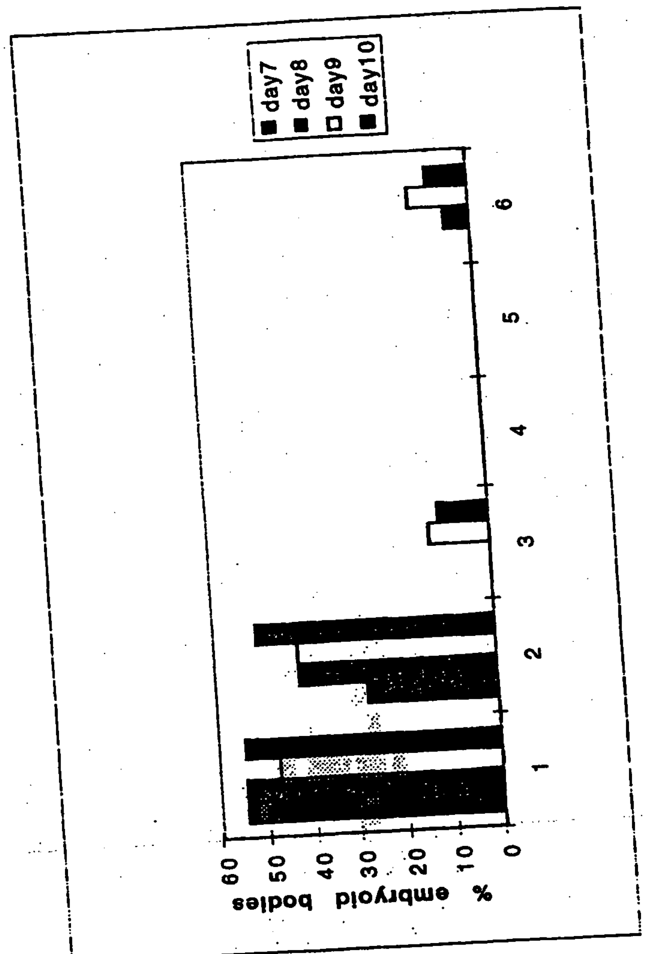

day7
day8
day9
day10
% embryoid bodies
60
50
40
30
20
10
0
1
2
3
4
5
6

FIGURE 36

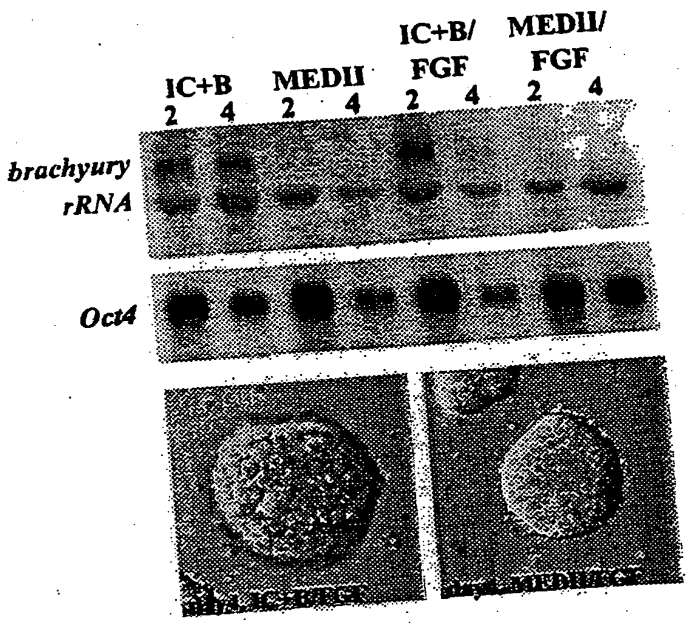


FIGURE 37

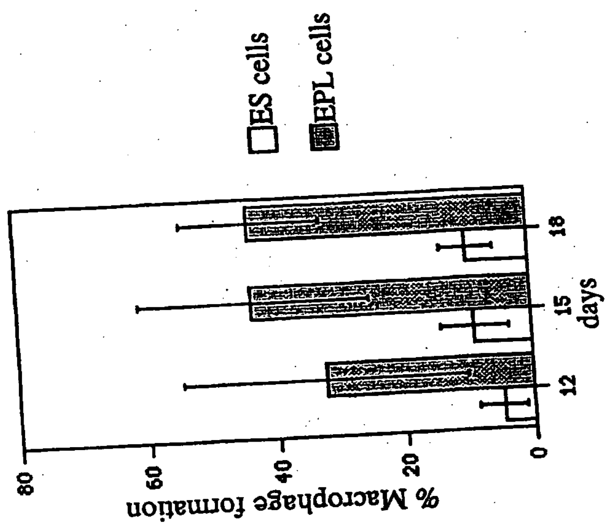
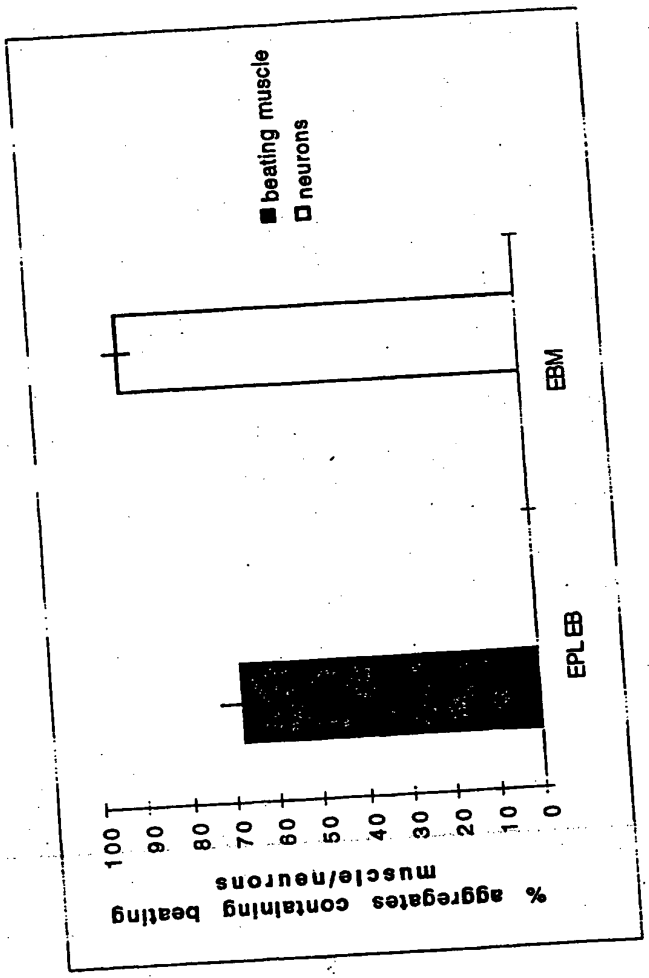

ES cells
EPL cells
% Macrophage formation
0
20
40
60
80
12
15
18
days

FIGURE 38


beating muscle
neurons
% aggregates containing beating muscle/neurons
100
90
80
70
60
50
40
30
20
10
0
EPL EB
EBM

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00265

A. CLASSIFICATION OF SUBJECT MATTER	
Int Cl[6]: C12N 1/38, 5/06, 5/16, 5/22 C07K 14/78	
According to International Patent Classification (IPC) or to both national classification and IPC	
B. FIELDS SEARCHED	
Minimum documentation searched (classification system followed by classification symbols) WPAT (SEE BELOW) CHEM ABS (SEE BELOW)	
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched MEDLINE (SEE BELOW)	
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) (Invention 1) WPAT: (((stem cell) or (pluripotent) or (extracellular matrix) or (matrix protein) or fibronectin) and (C12N 1/38 or C12N 5 or C07K 14/78) and proline) or ((stem cell) or (pluripotent)) and cont. suppl. sheet	

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	J. Cell Science 112, pages 601-12 (1999) Rathjen, J. et al "Formation of primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors." See entire document.	1-82
PX	Reprod. Fertil. Dev. 10, pages 31-47 (1998) Rathjen, P.D. et al "Properties and uses of embryonic stem cells: prospects for application to human biology and gene therapy." See entire document.	1-82
PX	WO A1 98/43679(THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE) 8 October 1998 See the entire document.	11, 28-30, 33, 35-40, 43-46, 49-57, 60-82

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search 17 May 1999	Date of mailing of the international search report 11 JUN 1999
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer TERRY MOORE Telephone No.: (02) 6283 2569

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Proc. Natl. Acad. Sci. USA 94, pages 5709-12 (1997) Brook, F.A. and Gardner, R.L. "The origin and efficient derivation of embryonic stem cells in the mouse." See materials and methods, table 1 and results.	36, 37, 77-82
X	Leukemia 3(Suppl.), pages 457-9 (1997) Kanatsu, M. et al "*In vitro* analysis of potency restriction during epiblast differentiation." See in particular figure 1.	1-7, 11,15-24, 28-32, 33, 35-44, 46, 47, 49, 51, 53
X	Development 122(3), pages 823-30 (1996) Kanatsu, M. and Nishikawa, S-I. "*In vitro* analysis of epiblast tissue potency for hematopoietic cell differentiation." See figures 1 & 2, table 1 and materials and methods.	1-7, 15-32, 35-45, 47, 49, 51, 53
X	US 5 453 357(VANDERBILT UNIVERSITY) 26 September 1995 See the entire document.	7, 28-30, 33, 35, 40, 43-47, 50-82
X	WO A1 95/00632 (AMGEN INC.) 5 January 1995 See in particular pages 8-10, 18, 20, 24-27	1-7, 11, 15-27
X	Blood 86(2), pages 463-72 (1995) Rich, I.N. "Primordial germ cells are capable of producing cells of the hematopoietic system *in vitro*." See entire document.	1-7, 11, 15-27
X	Molecular Reprod. Develop. 36, pages 139-47 (1993) Talbot, N.C. et al. "Alkaline phosphatase staining of pig and sheep epiblast cells in culture." See the introduction and discussion in particular.	7, 28-30, 35-40, 77-82
X	Biochem. Biophys. Res. Commun. 191(1), pages 188-95 (1993) Mummery, C.L. et al. "Fibroblast growth factor-mediated growth regulation and receptor expression in embryonal carcinoma and embryonic stem cells and human germ cell tumours." See the entire document.	8-11, 28, 29, 36-39, 43-47, 50-54
X	Differentiation 46, pages 51-60 (1991) Mummery, C.L. et al. "Visceral-endoderm-like cell lines induce differentiation of murine P19 embryonal carcinoma cells." See page 189 paragraphs 1 and 2 in particular.	7, 8, 11, 28, 29, 35-39, 43, 44, 46, 49, 51-54

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00265

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

See supplemental box..

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00265

Box supplemental

Cont. of box B.

((extracellular matrix) or (matrix protein) or fibronectin)
MEDLINE: ((stem cells/ct or hematopoietic stem cells/ct) or (primtive ectoderm) or (primitive endoderm) or (pluripotent(2n)cells) or (embryonic stem cell)) and ((extracellular matrix) or fibrinogen) and (tissue culture/ct or (conditioned medium))
CHEM ABS: ((hematopoietic precursor cell/ct or hematopoietic stem cell/ct) or (primitive ectoderm) or (primitive endoderm) or (pluripotent(2n)cell) or (embryonic stem cell)) and ((extracellular matrix) or fibrinogen) and ((in vivo) or (ex vivo) or tissue culture/ct or cell culture/ct))

(Invention 2) WPAT: (epiblast or (embryonic stem cell) or (primitive endoderm) or (primitive ectoderm) or pluripotent or (inner cell mass) or (embryonal carcinoma)) and C12N-005/IC
MEDLINE and CA: (conditioned(2n)medium) and (epiblast or (embryonic stem cell) or (primitive endoderm) or (primitive ectoderm) or (inner cell mass) or (embryonal carcinoma)

(Invention 3) WPAT: (primitive ectoderm) or epiblast or (inner cell mass) or (embryonic stem cell)
MEDLINE and CA: and ((primitive ectoderm) or epiblast) and ((tissue or cell or medium)2n(culture or cultured))

Cont of box II (unity of invention)

(1) Claims 1-7, 11, 15-35 and 38-76 define a biologically active factor consisting of proline or proline-containing peptides and/or a large molecular weight component consisting of extracellular matrix proteins.
(2) Claims 8-10 and 12-14 define a method for preparing conditioned medium or extracellular matrix using extra-embryonic cells or hepatic or hepatoma cells.
(3) Claims 36, 37 and 77-82 define cultured primitive ectoderm-like cells and methods of preparing nuclear transfer cells using EPL cells.

The only feature shared by all three sets of claims is the culture of pluripotent cells and the presence of factors essential to their differentiation or maintenance, however this feature is not novel and therefore does not constitute a special technical feature

International application No.
PCT/AU 99/00265

INTERNATIONAL SEARCH REPORT
Information on patent family members

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	95/00632	AU	71124/94	CA	2165335	EP	703978
WO	98/43679	AU	69477/98				

END OF ANNEX

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